CHAPTER 3

The Molecular Virology of Enteric Viruses

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1. CALICIVIRUSES: NOROVIRUSES AND SAPOVIRUSES

The *Caliciviridae* family is currently divided into five genera: *Norovirus*, *Sapovirus*, *Lagovirus*, *Vesivirus* and *Nebovirus* (King et al. 2012). Two other potential genera, *Valovirus* and *Recovirus*, have also been described (L'Homme et al. 2009b). The prototype norovirus, the Norwalk virus, was first described in 1972 as the etiological agent of an outbreak of acute gastroenteritis in an elementary school in Norwalk, Ohio (Kapikian 2000). Subsequently, Noroviruses (NoV) were found to be the cause of a majority of outbreaks of acute nonbacterial gastroenteritis. The NoVs are now recognized as a very common cause of sporadic cases of diarrhoea in the community (Glass et al. 2000b; Lopman et al. 2002; Hutson et al. 2004; Estes et al. 2006) and were found to be responsible for as many as 95 % of the reported viral gastroenteritis outbreaks over a 4.5-year period in the U.S. (Fankhauser et al. 2002). Similarly high percentages have also been reported from other industrialized countries (Maguire et al. 1999; Glass et al. 2000b; Koopmans et al. 2000; Lopman et al. 2003).

Common features of members of *Caliciviridae* include the presence of a single major structural protein from which the capsid is constructed and the appearance of 32 cup-shaped depressions on the surface of the virion arranged in an icosahedral symmetry. The name of the family was derived from the Latin word *calix,* which means cup or goblet, and refers to the surface hollows on the virion (Madeley 1979). Another major feature is the absence of a methylated cap structure or a ribosomal entry site (IRES) at the 5′ end of the viral RNA. Instead, a small protein (VPg) of ~10–12 kDa was shown to be covalently linked to the viral RNA and was described as essential for the infectivity of the RNA (Black et al. 1978). This has also been described for other caliciviruses (Burroughs and Brown 1978). The VPg protein interacts with components of the translation machinery (eIF3, eIFGI, eIF4E, and S6 ribosomal protein) and may play a role in initiating translation of NoV RNA (Daughenbaugh et al. 2003). The linkage of VPg to viral RNA is thought to occur during viral genome replication whereby VPg is attached as a protein primer to the 5′ terminus of the genomic RNAs (Rohayem et al. 2006).

The noroviruses and sapoviruses form distinct phylogenetic clades within the *Caliciviridae* family (Berke et al. 1997). In addition, certain features of their viral genome organization distinguish them from each other and from

S.M. Goyal, J.L. Cannon (eds.), *Viruses in Foods*, Food Microbiology and Food Safety, DOI 10.1007/978-3-319-30723-7_3

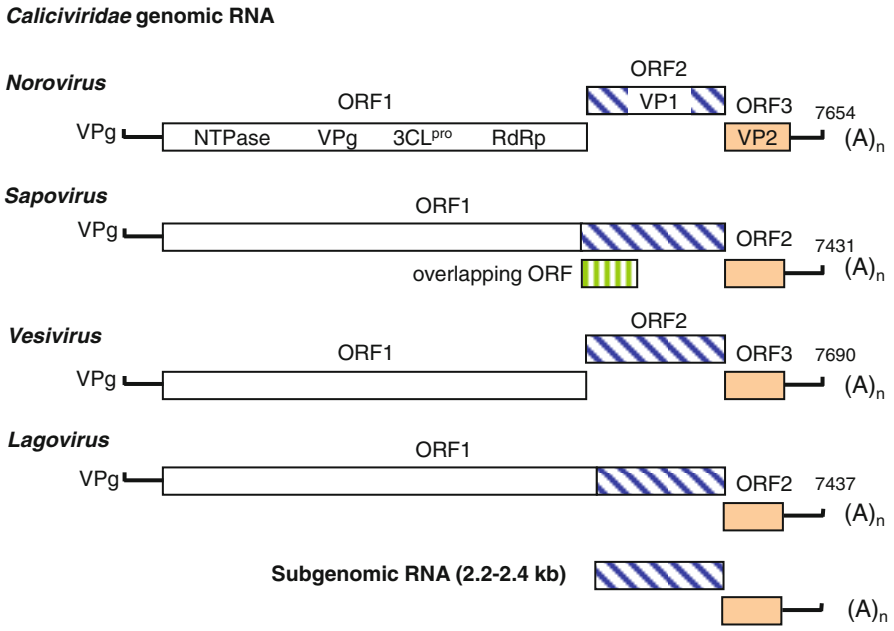

Figure 3.1. Genome organization of the four different genera of Caliciviridae. The genome of Norovirus and Vesivirus has three open reading frames (ORFs) that encode the nonstructural proteins, the major capsid protein (VP1), and a minor structural basic protein (VP2). The genera Sapovirus and Lagovirus encode the capsid protein contiguous with the large nonstructural polyprotein (ORF1). An additional small overlapping ORF in a +1 frameshift has been described in certain strains of sapoviruses. A subgenomic RNA that covers the entire 3′ end of the genome, from the capsid gene to the 3′ end, has been detected in calicivirus infected cells.

other genera of *Caliciviridae* (Fig. 3.1). Noroviruses and sapoviruses also differ in their epidemiology and host range. For example, NoVs can infect individuals of all ages and are commonly involved in outbreaks of acute gastroenteritis frequently associated with contaminated food or water. Sapoviruses, on the other hand, mainly infect infants and young children (Green et al. 2001) although gastroenteritis outbreaks in adults have also been described (Noel et al. 1997).

The NoVs have a polyadenylated positive-sense single-stranded RNA genome with three major open reading frames (ORFs) (Jiang et al. 1993b; Lambden et al. 1993). The virion has a buoyant density of 1.33–1.41 g/cm^3 in cesium chloride (CsCl) (Caul and Appleton 1982; Madore et al. 1986) and usually lacks the distinctive calicivirus cup-like morphology when viewed under electron microscope (EM). Noroviruses were formerly referred to as small round structured viruses (SRSVs). The sapoviruses, associated with sporadic cases of acute gastroenteritis, have a polyadenylated positive-sense

single-stranded RNA genome (7.3–7.5 kb in length) with two main ORFs (Liu et al. 1995). The virions have a buoyant density of 1.37–1.38 g/cm^3 in CsCl (Terashima et al. 1983), and often possess distinctive calicivirus cup-like morphology when viewed under EM (Madeley 1979). Sapoviruses ("classic" caliciviruses) are important enteric pathogens that can cause diarrhea in humans, pigs and mink (Guo et al. 1999b; Guo et al. 2001b; Martella et al. 2008).

Animal enteric caliciviruses are an important cause of gastroenteritis in domestic animals, namely calves and pigs (Liu et al. 1999; Saif et al. 1980; Bridger et al. 1984; van Der Poel et al. 2000; Guo et al. 2001a; Scipioni et al. 2008). Murine noroviruses (MNVs) have been isolated from both immunodeficient and immunocompetent laboratory mice (Karst et al. 2003; Hsu et al. 2006), but their pathogenicity is rather different from that caused by human noroviruses.

Despite numerous attempts, human noroviruses had not been propagated successfully in cultured cells until recently (Jones et al. 2014), thus hampering many aspects of research (Duizer et al. 2004; Herbst-Kralovetz et al. 2013; Papafragkou et al. 2013). However, considerable progress has been made by the analysis of cDNA clones generated from the genomic RNA of virions in stool material. In fact, the molecular era of norovirus research started with the successful cloning of the genomes of Norwalk and Southampton viruses from stool samples (Jiang et al. 1990; Lambden et al. 1993). Unlike human caliciviruses, some animal caliciviruses have been successfully propagated in cell cultures including primate calicivirus (Smith et al. 1983), feline calicivirus (FCV) (Love and Sabine 1975), and San Miguel sea lion virus (SMSV) (Smith et al. 1973). These viruses have provided a direct approach for the study of virus infections, genome transcription, viral protein translation, and virus replication (Green et al. 2002). In addition, information gained by the study of caliciviruses that grow efficiently in cell culture, such as FCV and vesicular exanthema of swine virus (VESV), or that have an animal model and a limited cell culture system such as rabbit hemorrhagic disease virus (RHDV), has been important for the identification of features that are likely to be shared among members of the *Caliciviridae* family (Marin et al. 2000; Morales et al. 2004).

Thus, the murine norovirus (MNV) revealed an unexpected tropism for hematopoietic cell lineages, particularly dendritic cells and macrophages (Wobus et al. 2004). The MNV can be grown in vitro; the RAW264.7 cell line has been used to design a typical virus plaque assay and to grow plaque-purified viral isolates (Mumphrey et al. 2007). The MNV has offered the first chance to study the entire norovirus replication cycle in the laboratory. Despite differences in diseases caused by human and murine NoVs, the MNV possesses a high genetic similarity and constitute an excellent model system to study the mechanisms of NoV translation and replication, as well as pathogenesis and immunity (Vashist et al. 2009). More recently, Jones et al. showed that human noroviruses could be propagated in the human B lymphocyte derived cell line BJAB (Jones et al. 2014). These experiments confirm the tropism of noroviruses for hematopoietic cell lineages. Another relevant aspect of norovirus

infections is the role of gut microbiota as a cofactor needed for efficient virus-cell attachment and infection, as it has been demonstrated "in vitro" for human noroviruses (Miura et al. 2013; Rubio-del-Campo et al. 2014; Jones et al. 2014) and "in vivo" for murine norovirus (Jones et al. 2014; Baldridge et al. 2015).

1.1. Structure and Composition

The Norwalk virus capsid is composed of a single major structural protein known as VP1, and a few copies of a second small basic structural protein named as VP2 (Prasad et al. 1999; Glass et al. 2000a; Green et al. 2001). The characteristic cup-shaped structures on the surface of the virions are more prominent in some strains, particularly in sapoviruses, leading to the characteristic six-pointed "star of David" appearance when viewed along the major two-, three- and fivefold axes of symmetry. Cloning and expression of norovirus proteins VP1 and VP2 in insect cells using the baculovirus expression system resulted in the self-assembly of the viral capsid and the production of recombinant virus-like particles (rVLPs) that were antigenically and structurally similar to native virions (Jiang et al. 1992; Green et al. 1997; Hale et al. 1999; Kobayashi et al. 2000).

The three-dimensional structure of Norwalk rVLPs was first determined by cryo-electron microscopy and computer image processing to a resolution of 22 Å. This analysis showed that the virus particles (38 nm in diameter by this technique) have a distinct architecture and exhibit T = 3 icosahedral symmetry (Fig. 3.2). The capsid contains 180 copies of the capsid protein assembled into 90 dimers with an arch-like structure. The arches are arranged in such a way that there are large hollows at the icosahedral five- and threefold positions; these hollows are seen as cuplike structures on the surface of caliciviruses (Prasad et al. 1994; Prasad et al. 1996a; Prasad et al. 1999). To form a T = 3 icosahedral structure, the capsid protein has to adapt to three quasi-equivalent positions; the subunits located at these positions are conventionally referred to as A, B and C. The high resolution (3.4 Å) structure of the Norwalk virus capsid has been determined by X-ray crystallography (Prasad et al. 1999).

Each subunit or monomeric capsid protein folds into an N-terminal region facing the inside of the capsid, a shell-domain (S) that forms the continuous surface of the VLP, and a protruding (P) domain that forms the protrusions (Fig. 3.2). A flexible hinge of eight amino acids links the S and P domains. The P domain is located at the exterior of the capsid and is likely to contain determinants of genotype specificity. The NH_2-terminal (N) arm, located within the S domain, consists of residues 10–49 and faces the interior of the capsid. The part of the S domain that forms a ß-barrel consists of amino acids 50–225. The entire S domain (amino acids 1–225) corresponds to the N-terminal region of the capsid protein that is relatively conserved among "Norwalk-like viruses" in sequence comparisons. Amino acid residues 226–530 form the P domain, which corresponds to the C-terminal half of the capsid protein and forms the arch-like structures extending from the shell. The S domain is

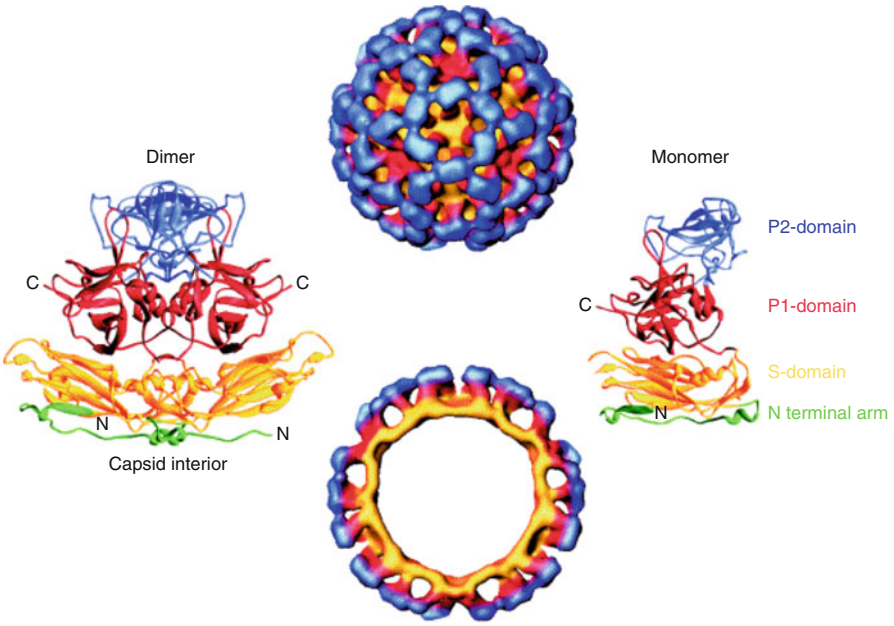

Figure 3.2. The structure of Norwalk virus-like particles (NV VLPs) has been solved by cryo-electron microscopic reconstruction to 22 Å (top, surface representation; bottom, cross section) and by x-ray crystallography to 3.4 Å. The NV VLPs have 90 dimers of capsid protein (*left*, ribbon diagram) assembled in T = 3 icosahedral symmetry. Each monomeric capsid protein (*right*, ribbon diagram) is divided into an N-terminal arm region (*green*) facing the interior of the VLP, a shell domain (S domain, *yellow*) that forms the continuous surface of the VLP, and a protruding domain (P domain) that emanates from the S domain surface. The P domain is further divided into subdomains, P1 and P2 (*red* and *blue*, respectively) with the P2 subdomain at the most distal surface of the VLPs (Reproduced with permission from Prasad et al. 1999, and Bertolotti-Ciarlet et al. 2002).

required for the assembly of the capsid and also participates in multiple intermolecular interactions of dimers, trimers and pentamers.

The P domain is mainly involved in dimeric interactions only (Prasad et al. 1999) and has two subdomains: P1 formed by amino acids 226–278 and 406–530, and P2 encompassing amino acids 279–405. The P2 subdomain is the most variable region of the capsid protein among NoVs (Hardy et al. 1996) and plays an important role in immune recognition and receptor interaction. It has been shown that isolated P domains form dimers and bind to histo-blood group antigens (HBGA) without requiring the formation of VLPs (Tan et al. 2004). In addition, a binding pocket in the P domain is responsible for viral HBGA-binding and the formation of this pocket involves only intramolecular interactions (Tan et al. 2003). Although the S domain has a canonical 8-stranded β-barrel structure, the P2 subdomain has a fold similar to that observed in

domain 2 of the elongation factor Tu (EF-Tu), a structure never seen before in a viral capsid protein (Prasad et al. 1999). Moreover, the fold of the P1 subdomain is unlike any other polypeptide observed so far (Bertolotti-Ciarlet et al. 2003).

A comparison of the capsid proteins from various caliciviruses reveals significant variations in their sequences and sizes. In general, the capsid proteins of human caliciviruses are smaller than those of animal caliciviruses (Chen et al. 2004). The Norwalk virus recombinant capsid protein can also self-assemble into smaller VLPs (23 nm) with suspected T = 1 symmetry that are thought to be composed of 60 copies of the capsid protein (White et al. 1997).

It has been postulated that the N-terminal region of the capsid protein or the genomic RNA acts as a switching region that controls variations in the conformation of the coat protein of the T = 3 viruses (Rossmann and Johnson 1989). NoV particles are different from other T = 3 viruses; their recombinant capsid protein readily forms rVLPs without RNA (Prasad et al. 1999). It has been suggested that the determinants for the T = 3 capsid assembly for Norwalk virus may lie outside of the N-terminus and that the interaction between the subunits B and C is not mandatory for the formation of the capsid (Bertolotti-Ciarlet et al. 2002).

1.2. Genomes and Proteins

As mentioned above, all caliciviruses have a linear, single-stranded, positive-sense RNA genome of 7.5–7.7 kb (Green et al. 2001) (Fig. 3.1). The RNA genome of Norwalk virus, the prototype strain for the genus *Norovirus*, is 7654 nucleotides in length and is polyadenylated at the 3′ end (Jiang et al. 1993b). The lack of a cap structure typical of eukaryotic mRNA and the absence of an internal ribosomal entry site suggest that the VPg protein may function in translation initiation through unique protein-protein interactions with the cellular translation machinery (Daughenbaugh et al. 2003). The genomes of caliciviruses are organized into either two or three major ORFs with the exception of MNV, which has a fourth alternative ORF (McFadden et al. 2011). The nonstructural proteins encoded in the calicivirus ORF1 were first predicted based on sequence similarities with picornavirus nonstructural proteins (Neill 1990). Amino acid sequence motifs in common with the poliovirus 2C NTPase, 3C protease, and 3D RNA-dependent RNA polymerase (RdRp) were readily identified and provided templates for further characterization of the calicivirus nonstructural proteins. Proteolytic mapping and enzymatic studies of in vitro-translated polyprotein or recombinant protein expression has confirmed the presence of an NTPase (p41), a 3C-like protease ($3CL^{pro}$), an RdRp, and the location in the polyprotein of the genome-linked protein VPg (Liu et al. 1996; Dunham et al. 1998; Pfister and Wimmer 2001). The proposed six nonstructural proteins encoded in the norovirus ORF1 defined so far proceed from N to C terminus, p48-NTPase-p22-VPg-$3CL^{pro}$-RdRp (Ettayebi and Hardy 2003). It has been reported that the 3C-like proteinase ($3CL^{pro}$) inhibits host cell translation by cleavage of poly(A)-binding protein (PABP), which is a key protein involved in the translation of

polyadenylated mRNAs (Kuyumcu-Martinez et al. 2004). Functional analysis of the protein produced from MNV ORF4 demonstrated that it antagonizes the innate immune response to infection by delaying the upregulation of a number of cellular genes activated by the innate pathway, including IFN-beta (McFadden et al. 2011).

In the genera *Norovirus* and *Vesivirus*, the capsid structural protein VP1 is encoded in a separate ORF (ORF2), whereas the capsid proteins encoded in *Sapovirus* and *Lagovirus* genera are contiguous with the large nonstructural polyprotein (ORF1) (see Fig. 3.1). Viruses in the genera *Sapovirus* and *Lagovirus* have two major ORFs (ORF1 and ORF2). In the *Sapovirus* genera, the ORF1/2 junction consists of a one- or four-nucleotide overlap between the stop codon of ORF1 and the first AUG codon of ORF2. A third ORF has been described in certain strains that overlaps with the capsid protein gene in a +1 frameshift and is not found in the *Norovirus* genome (Liu et al. 1995; Clarke and Lambden 2000). The presence of a conserved translation initiation motif GCAAUGG at the 5′ end of this overlapping ORF suggests that a biologically active protein may be encoded in this ORF (Schuffenecker et al. 2001).

Viruses in the genera *Norovirus* and *Vesivirus* have three major ORFs (ORF1, ORF2, and ORF3). In the noroviruses the first and third ORFs are in the same reading frame. In Norwalk virus, ORF3 encodes a 212-amino-acid minor structural protein of the virion (Glass et al. 2000a). All calicivirus genomes begin with a 5′-end terminal GU, which is repeated internally in the genome and most likely correspond to the beginning of a subgenomic-sized RNA transcript (2.2–2.4 kb) that is co-terminal with the 3′ end of the genome. This has been observed in FCV- and RHDV-infected cells as well as in packaged virions (Herbert et al. 1996a). A comparison of the 5′-end sequences of representative viruses within each of the four genera and the corresponding repeated internal sequence suggests this feature is characteristic of the caliciviruses. The synthesis of a subgenomic RNA in calicivirus-infected cells is a major difference between the replication strategy of caliciviruses and picornaviruses, although several of the replicative enzymes share distant homology (Green et al. 2001). Calicivirus structural proteins are expressed from a subgenomic mRNA with two overlapping cistrons. The first ORF of this RNA codes for the major capsid protein VP1, while the second codes for the minor capsid protein VP2. Translation of VP2 is mediated by a termination/reinitiation mechanism, which depends on an upstream sequence element of approximately 70 nucleotides denoted "termination upstream ribosomal binding site" (TURBS). Two short sequence motifs within the TURBS were found to be essential for reinitiation (Luttermann and Meyers 2009).

By functional analysis of the 5′ genomic sequence of Jena virus, a bovine norovirus, the N-terminal encoding genomic sequence was tested for IRES-like function in a bi-cistronic system but displayed no evidence of IRES-like activity (Salim et al. 2008). Alignment of the N-terminal protein sequences of various noroviruses showed little similarity between genogroups within the first 180 residues; however, towards the C-terminal end of the protein,

similarity between the amino acid residues increased. Recent studies investigating the functions of the Norwalk virus N-terminal protein have successfully demonstrated its association with the Golgi apparatus in transfected cells (Fernandez-Vega et al. 2004). Other studies have suggested that the Norwalk virus N-terminal protein disrupts intracellular protein trafficking, including proteins destined for the host cell membrane (Ettayebi and Hardy 2003). A 3C protease-mediated cleavage event within the N-terminal protein (37 kDa) was described for Camberwell virus, a genogroup 2 norovirus, yielding proteins of 22 and 15 kDa (Seah et al. 2003). Based on these observations and location within the genome it was hypothesised that the N-terminal protein of noroviruses corresponds to the 2AB region in picornaviruses. After expression of the structural proteins from subgenomic RNA molecules, the capsid is assembled and viral RNA is encapsidated prior to progeny release. Some of these features have been confirmed using recombinant systems to express the native Norwalk virus genomes in mammalian cells using vaccinia virus expression systems (Asanaka et al. 2005; Katayama et al. 2006).

1.3. Molecular Diversity of Noroviruses

Early studies demonstrating variability in NoVs soon led to the notion that it was important to distinguish between strains to better understand their epidemiology. Since no antigenic analysis of norovirus strains was available due to the lack of immune reagents, genome characterization by sequence analysis has been used to provide an interim system of genotyping (Koopmans et al. 2003). As the genotypes ideally would correlate with serotypes, the amino acid sequence of the major structural protein was used as the basis for phylogenetic analyses (Ando et al. 2000; Koopmans et al. 2001). Noroviruses encompass six distinct genogroups (GI-GVI) with GI, GII and GIV infecting humans, which are further divided into >30 genotypes (Zheng et al. 2006; Vinjé 2015) (Table 3.1). Genogroups were defined on the basis of amino acid diversity of the 3 ORFs, the RdRp and VP1 encoding regions, or the VP1 encoding region alone. Current classification into six genogroups is based on the VP1 protein divergence (Green et al. 2000; Vinjé et al. 2004; Zheng et al. 2006; Vinjé 2015). Genotypes are defined on the basis of either the polymerase or the capsid sequence, assuming that the genotype defined by one region of the genome would correspond to the whole genome because of the non-segmented nature of the norovirus genome (Vinjé and Koopmans 1996; Kageyama et al. 2004; Vinjé et al. 2004). New genotypes were assigned when VP1 amino acid sequence differed by more than 20 % with other known genotypes (Vinjé et al. 2000). However, with the accumulation of more norovirus sequence data, this cutoff threshold has been changed to 14.1 % and a minimum of 15 % pairwise difference between the next-nearest genotype for the proposal of a new norovirus genotype (Zheng et al. 2006; Kroneman et al. 2013).

The recognition that recombination often occurs at the ORF1/ORF2 junction, has necessitated the determination of both polymerase and capsid gene

Table 3.1 Current genogroups and genotypes of noroviruses (adapted from Green et al. (2001), Koopmans et al. (2003) and Vinjé (2015))

Genogroup	*Genotype*	*Prototype strain*[a]	*GenBank accession number (capsid gene)*	*Other strains*
I	1	Norwalk/1968/UK	M87661	KY/89/JP
	2	Southampton/1991/UK	L07418	White Rose, Crawley
	3	Desert Shield/395/1990/SA	U04469	Birmingham 291
	4	Chiba 407/1987/JP	D38547	Thistle Hall; Valetta, Malta
	5	Musgrove/1989/UK	AJ277614	Butlins
	6	Hesse 3/1997/DE	AF093797	Sindlesham, Mikkeli, Lord Harris
	7	Winchester/1994/UK	AJ277609	Lwymontley
	8	Boxer/2001/US	AF538679	
	9	Vancouver730/2004/CA	HQ637267	
II	1	Hawaii/1971/US	U07611	Wortley, Girlington
	2	Melksham/1994/UK	X81879	Snow Mountain
	3	Toronto 24/1991/CA	U02030	Mexico, Auckland, Rotterdam
	4	Bristol/1993/UK	X76716	Lordsdale, Camberwell, Grimsby, New Orleans, Sydney
	5	Hillingdon/1990/UK	AJ277607	White river
	6	Seacroft/1990/UK	AJ277620	
	7	Leeds/1990/UK	AJ277608	Gwynedd, Venlo, Creche
	8	Amsterdam/1998/NL	AF195848	
	9	VA97207/1997/US	AY038599	
	10	Erfurt546/2000/DE	AF427118	
	11	Sw918/1997/JP	AB074893	
	12	Wortley/1990/UK	AJ277618	
	13	Fayetteville/1998/US	AY113106	
	14	M7/1999/US	AY130761	
	15	J23/1999/US	AY130762	
	16	Tiffin/1999/US	AY502010	
	17	CS-E1/2002/US	AY502009	
	18	OH-QW101/2003/US	AY82330	
	19	OH-QW170/2003/US	AY823306	

(continued)

Table 3.1 (continued)

Geno-group	*Geno-type*	*Prototype strain*[a]	*GenBank accession number (capsid gene)*	*Other strains*
	20	Luckenwalde591/2002/DE	EU373815	
	21	IF1998/2002/IQ	AY675554	
	22	Yuri/2003/JP	AB083780	
III	1	Jena/1980/DE	AJ011099	Bovine strains
IV	1	Alphatron/1998/NL	AF195847	Ft. Lauderdale
V	1	MNV-1/2002/US	AY228235	Murine strains
VI	1	Bari91/2007/IT	FJ875027	

[a]*CA* Canada, *DE* Germany, *IQ* Iraq, *IT* Italy, *JP* Japan, *NL* Netherlands, *SA* Saudi Arabia, *UK* United Kingdom, *US* United States

sequences to fully describe the genotypic features of clinical strains (Buesa et al. 2002; Bull et al. 2007; Hoa Tran et al. 2013). Recombination is a potentially important means by which these viruses generate diversity (Bull et al. 2007; Eden et al. 2013). Dual genotyping based on the polymerase encoding region and the capsid amino acid sequence can be conducted by using a web-based tool: http://www.rivm.nl/mpf/norovirus/typingtool (Kroneman et al. 2011). The method employs percent pairwise similarities in reference to prototype strains. Thus, the polymerase genotype is defined with a cut-off value of ≥15 % for the polymerase nucleotide sequence (about 800 nt at the 3′ end of ORF1), whereas the capsid genotype is defined with a cut-off value of ≥15 % for the major capsid protein (VP1) amino acid sequence (Vinjé et al. 2004; Zheng et al. 2006). Thus far, 14 GI and 29 GII polymerase genotypes and 9 GI and 22 GII capsid genotypes have been described (RIVM-NoroNet) (Kroneman et al. 2013). Genotype GII.4 is further divided into variants (subgenotypes) based on sequence diversity (Vinjé et al. 2004; Siebenga et al. 2007; Buesa et al. 2008; Kroneman et al. 2011). New GII.4 variants are recognized only after evidence is provided that they have become epidemic lineages in at least two geographically diverse locations. Table 3.2 shows the new consensus nomenclature for epidemic norovirus GII.4 variants (Kroneman et al. 2013).

Molecular characterization of bovine enteric caliciviruses suggests their inclusion into GIII, which contains viruses so far found only in cattle (Ando et al. 2000; Oliver et al. 2003; Smiley et al. 2003). Phylogenetic analysis places at least two human noroviruses within genogroup IV: strains Alphatron (GenBank accession number AF195847) and Ft. Lauderdale (GenBank accession number AF414426) (Fankhauser et al. 2002). The porcine noroviruses cluster within GII (Sugieda and Nakajima 2002). The murine noroviruses have been included into GV (GenBank accession number DQ285629), members of which are closer to GII than those of GI by sequence alignment (Karst et al. 2003). In the

Table 3.2 Proposed epidemic norovirus GII.4 variants (adapted from Kroneman et al. (2013) and Kroneman et al. (2011))

Proposed epidemic variant name	*GenBank no.*[a]
US95_96	AJ004864[b]
Farmington_Hills_2002	AY485642[c]
Asia_2003[d]	AB220921[c]
Hunter_2004	AY883096[b]
Yerseke_2006a	EF126963[b]
Den Haag_2006b	EF126965[b]
NewOrleans_2009	GU445325[c]
Sydney_2012[e]	JX459908[c]

[a]GenBank accession number of the first submitted capsid sequence of this variant
[b]Capsid sequence
[c]Complete genome
[d]Variant Asia_2003 is a recombinant with a GII.4 ORF2 and a GII.P12 ORF1
[e]Variant Sydney_2012 is a recombinant with a GII.4 ORF2 and a GII.Pe ORF1

major capsid protein VP1, human norovirus strains within the same genogroup share at least 60 % amino acid sequence identity, whereas most GI strains share less than 50 % amino acid identity with GII strains (Green et al. 2001; Koopmans et al. 2003). Picornavirus serotypes generally have >85 % amino acid identity across the VP1 gene, which is in the range of the cut-off for calicivirus genotypes (>80 % amino acid identity) (Oberste et al. 1999).

Caliciviruses contain subgenomic RNA encoding regions that span from the start of the capsid gene to the 3′ end (Fig. 3.1). It has been hypothesized that the subgenomic RNA could act as an independent unit participating in recombination events. If RNA recombination is a common phenomenon among caliciviruses, high diversity of the family might be expected, which would facilitate the emergence of new variants and make genotyping more difficult (Bull et al. 2012). Recombination may permit caliciviruses to escape host immunity, analogous to antigenic shifts by influenza viruses, but by a different molecular mechanism. It has also been reported that accumulation of mutations in the protruding P2 domain of the capsid protein may result in predicted structural changes, including disappearance of a helix structure of the protein, and thus a possible emergence of new phenotypes (Nilsson et al. 2003; Lindesmith et al. 2011).

1.4. Genetic Classification of Sapoviruses

The sapovirus major capsid protein-encoding region is fused to and in frame with the polyprotein encoding region (Lambden et al. 1994; Numata et al. 1997). All published sapovirus strains except a human strain, London/92, and

Table 3.3 Classification of current genogroups and genotypes of sapoviruses (adapted from Schuffenecker et al. (2001) and Farkas et al. (2004))

Genogroup/ cluster	*Prototype strain*	*GenBank accession number (capsid gene)*	*Other strains*
GI/1	Sapporo/82	U65427	Houston/86, Plymouth/92, Manchester/93, Lyon30388/98
GI/2	Parkville/94	U73124	Houston/90
GI/3	Stockholm/97	AF194182	Mexico14917/00
GII/1	London/92	U95645	Lyon/598/97
GII/2	Mexico340/90	AF435812	
GII/3	Cruise ship/00	AY289804	
GIII	PEC/Cowden	AF182760	
GIV	Houston7-1181/90	AF435814	
GV	Argentina39	AY289803	

a porcine strain, PEC Cowden, contain an additional ORF predicted in +1 frame, overlapping the N terminus of the capsid encoding region (Guo et al. 1999b; Jiang et al. 1999; Clarke and Lambden 2001). By analogy with the classification of the noroviruses, the sapoviruses were previously divided into four genotypes, belonging to two genogroups (Liu et al. 1995; Noel et al. 1997; Clarke and Lambden 2000). More recently, human sapoviruses have been classified into five genogroups (Schuffenecker et al. 2001; Farkas et al. 2004) (Table 3.3). In addition, the porcine enteric calicivirus (Cowden strain) has been shown to be related to the *Sapovirus* genus but belongs to a differentiated cluster (Guo et al. 1999b). Further classification of sapovirus into genotypes has also been undertaken, though taxonomic assignment at the genotype level appears to be less well-defined than at the genogroup level (L'Homme et al. 2010; Oka et al. 2012). Members of SaV GI, GII, GIV, and GV are found to infect humans and those of GIII are found in swine (Farkas et al. 2004). Each genogroup can be further subdivided into a number of genotypes or genetic clusters. Recent studies underscore the ambiguity in classification of animal SaVs beyond the species level and serves as a caution against proposing novel *Sapovirus* genogroups based only on short polymerase sequences (L'Homme et al. 2009a).

1.5. Virus Replication

Studies on the replication strategy of human caliciviruses have been hampered by the lack of an efficient cell culture system or an appropriate animal model. Nevertheless, the first mouse model for HuNoVs has recently been reported; it consists of BALB/c mice deficient in recombination activation gene (Rag) 1 or 2 and common gamma chain (γc) ($Rag^{-/-}\gamma c^{-/-}$ mice) (Taube et al. 2013). If validated, this model has the potential to accelerate our

knowledge of human norovirus biology and help investigate the molecular mechanisms regulating norovirus infections.

Expression of recombinant proteins from cDNA clones has allowed the generation of proteolytic processing maps for the nonstructural proteins of several caliciviruses e.g., Southampton virus (a norovirus) and RHDV (a lagovirus) (Liu et al. 1996; Wirblich et al. 1996). Analysis of individual recombinant proteins from these noncultivable caliciviruses allowed the identification of NTPase and 3C-like cysteine protease activities for RHDV and noroviruses (Liu et al. 1999) and a 3D-like RNA-dependent RNA polymerase for RHDV (López-Vazquez et al. 1998). Studies on the replication mechanisms of cultivatable caliciviruses, like feline calicivirus (FCV), have contributed to a better understanding of the basic features of calicivirus replication (Sosnovtsev and Green 2003). The FCV replicates by producing two major types of polyadenylated RNAs; a positive-sense genomic RNA of approximately 7.7 kb, and a subgenomic RNA of 2.4 kb (Herbert et al. 1996b). The genomic RNA serves as a template for synthesis of the nonstructural protein encoded by the ORF1, whereas the subgenomic RNA is a template for translation of structural proteins (Carter 1990).

Guix et al. (2007) demonstrated that transfection of Norwalk virus RNA into human hepatoma Huh-7 cells leads to viral replication with expression of viral antigens, RNA replication, and release of viral particles into the medium. Prior treatment of RNA with proteinase K completely abolished RNA infectivity, suggesting a key role of a RNA-protein complex. However, a block to viral spread to other cells in the culture remained, indicating that the blockade exists at the stage of cell entry and/or uncoating (Guix et al. 2007). A number of host factors are important in norovirus life cycle as demonstrated by proteomics and reverse genetics (Bailey et al. 2010; Yunus et al. 2010). It has been shown that the norovirus VPg protein interacts with the host cell cap-binding proteins eIF4E and eIF4G (Chaudhry et al. 2007). The crystallographic structures of the VPg proteins from FCV and MNV have been recently determined by nuclear magnetic resonance spectroscopy (Leen et al. 2013).

Reverse genetics and replicon systems have become important tools to elucidate the mechanism of calicivirus replication and pathogenicity. Reverse genetics systems are available for cultivatable viruses such as FCV (Sosnovtsev and Green 1995), MNV (Ward et al. 2007), porcine enteric calicivirus (Chang et al. 2005), RHDV (Liu et al. 2008) and Tulane virus, a rhesus monkey calicivirus (Wei et al. 2008). For uncultivatable caliciviruses, such as human norovirus, replicon systems with either transient or stable expression of viral RNA have been developed. Transfection of a full-length cDNA clone of the Norwalk virus RNA under the control of T7 promoter into MVA-T7 infected cells allowed the expression of viral proteins and Norwalk virus RNA replication (Asanaka et al. 2005). A cell-based replicon was generated for Norwalk virus replication using a cloned cDNA consensus sequence of the Norwalk virus genome (Fernandez-Vega et al. 2004) engineered to encode the neomycin resistance gene as a selective marker within ORF2. However, only few

transfected cells could apparently support virus replication, suggesting that severe growth restrictions were present in the cells (Chang et al. 2006; Chang et al. 2008).

1.6. Virus-Cell Interactions

Human and animal enteric caliciviruses are assumed to replicate in the upper intestinal tract, causing cytolytic infections in the villous enterocytes but not in the crypt enterocytes of the proximal small intestine. Biopsies of the jejunum taken from experimentally infected volunteers who developed gastrointestinal disease following oral administration of noroviruses showed histopathologic lesions consisting of: blunting of the villi, crypt cell hyperplasia, infiltration with mononuclear cells, and cytoplasmic vacuolization (Blacklow et al. 1972; Dolin et al. 1975). Experiments with recombinant Norwalk VLPs and human gastrointestinal biopsies showed that VLPs bind to epithelial cells of the pyloric region of the stomach and to enterocytes on duodenal villi. Attachment of the rVLPs occurred only on cells as well as to saliva from histo-blood group antigen (HBGA)-secreting individuals (Marionneau et al. 2002). It was previously determined that RHDV attaches to H type 2 HBGAs present on rabbit epithelial cells (Ruvöen-Clouet et al. 2000). Significant attachment of Norwalk rVLPs onto differentiated Caco-2 cells has been demonstrated (White et al. 1996). Differentiated Caco-2 cells, derived from an individual expressing the group O blood type, resemble mature enterocytes and express H-type HBGAs on their cell surfaces (Amano and Oshima 1999).

To date, the Cowden strain of porcine enteric calicivirus (PEC) is the only cultivatable enteric sapovirus (Flynn and Saif 1988). However, for replication, it requires the incorporation of an intestinal contents preparation (ICP) from uninfected gnotobiotic pigs or bile acids as a medium supplement (Chang et al. 2004). Different porcine intestinal enzymes such as trypsin, pancreatin, alkaline phosphatase, enterokinase, elastase, protease and lipase were tested as medium supplements, but none of them alone promoted viral growth in cell culture (Parwani et al. 1991). It was speculated that some enzymes or factors in the porcine ICP could activate the viral receptor, promote signalling of host cells, or might help in cleavage of the viral capsid for successful uncoating (Guo and Saif 2003).

Although noroviruses are highly infectious (it has been estimated that as low as 18 and as high as 2800 virions are enough to infect an adult (Atmar et al. 2014; Teunis et al. 2008) studies in volunteers have shown that some subjects remain uninfected despite having been challenged with a high dose of virus (Matsui and Greenberg 2000). This could be due to the presence of innate resistance or pre-existing immunity to the virus (Lindesmith et al. 2003). An increased risk of Norwalk virus infection has been associated with blood group O; and Norwalk VLPs bind to gastroduodenal cells from individuals who are secretors (Se+) but not to those from non-secretors (Se-) (Hutson et al. 2002; Marionneau et al. 2002). The gene responsible for the

secretor phenotype *FUT2* encodes an α(1,2) fucosyltransferase that produces H type HBGAs found on the surface of gastrointestinal epithelial cells and in mucosal secretions (Lindesmith et al. 2003). Additional forms of HBGAs found on the gut mucosa and its secretions depend on additional glycosyltransferases, including those that produce the Lewis antigens and the A- and B-type antigens related to those present on red blood cells (Marionneau et al. 2001). The discovery that noroviruses attach to cells in the gut only if the individual expresses specific, genetically-determined HBGA carbohydrates is a breakthrough in understanding norovirus-host interactions and susceptibility to norovirus infections (Lindesmith et al. 2003; Hutson et al. 2004). Individuals with defects in the *FUT2* gene are termed secretor-negative, do not express the appropriate HBGAs necessary for Norwalk virus binding, and are therefore resistant to Norwalk virus infection. These data argue that *FUT2* and other genes encoding enzymes that regulate the processing of the HBGA carbohydrates function as susceptibility alleles. However, secretor-negative individuals can be infected with other norovirus strains (Le Pendu et al. 2006; Lindesmith et al. 2008).

To explore the process of norovirus attachment to cells, virus-like particles (VLPs), which mimic native virions morphologically and antigenically, have been used (White et al. 1996; Lindesmith et al. 2003; Allen et al. 2008). The C-terminal of the P-domain of NoVs has the ability to auto-assemble into subviral particles, termed P particles (Tan and Jiang 2005), which are potentially useful for broad applications including vaccine development (Tan et al. 2011). The C-terminal region (P domain) of the capsid protein is involved in HBGA attachment and, therefore, plays an essential role in cell infection (Marionneau et al. 2002; Hutson et al. 2003). The crystal structures of the HBGA-binding interfaces of Norwalk virus (GI.1) and VA387 (GII.4) have been elucidated, each representing one of the two major genogroups of human noroviruses (Cao et al. 2007; Choi et al. 2008). The HBGA-binding interfaces of the two strains differ significantly in their structures, precise locations of HBGA-binding locations and amino acid compositions, although both locate on the top of the arch-like P dimer of the viral capsids (Tan et al. 2009). Sequence alignment has shown that the key residues responsible for HBGA binding are highly conserved within but not between genogroups GI and GII and the remaining sequences of the P2 subdomain are highly variable (Tan et al. 2009; Tan and Jiang 2010). It has been suggested that noroviruses may use secondary or alternative receptors other than HBGAs when attaching to enterocytes. Hence HBGAs could play a role as co-receptors but blocking such interactions may not abolish attachment to these cells (Murakami et al. 2013).

Recent studies also suggest that some animal caliciviruses may cross the species barrier and potentially infect humans. The hypothetical existence of animal reservoirs and the possibility of interspecies transmission have been suggested by phylogenetic linkage between human and bovine or porcine viruses within the genera *Norovirus* and *Sapovirus*, respectively (Clarke and

Lambden 1997; Dastjerdi et al. 1999; Liu et al. 1999; van Der Poel et al. 2000). However, information concerning the frequency of interspecies transmission among caliciviruses is limited. Given the genetic similarity of human and animal caliciviruses and their potential for recombination, interspecies transmission of noroviruses and sapoviruses is possible, although not demonstrated to date.

2. ROTAVIRUSES

Rotaviruses are the leading cause of viral gastroenteritis in infants and young children worldwide and also in the young of a large variety of animal species (Parashar et al. 2003; Parashar et al. 2006). Rotavirus infections in humans continue to occur throughout their lives but the resulting disease is mild and often asymptomatic (Bishop 1996). In addition to sporadic cases of acute gastroenteritis, outbreaks of rotavirus diarrhea in school-aged children and adults have increasingly been reported (Griffin et al. 2002; Mikami et al. 2004; Rubilar-Abreu et al. 2005). Rotaviruses are known to produce disease in humans since 1973 (Bishop et al. 1973; Flewett et al. 1973a). They are responsible for an estimated 500,000 deaths each year, mostly in infants and young children in developing countries (Parashar et al. 2009). The main strategy to combat rotavirus infection has been the development of rotavirus vaccines. Since the 1980s this has been the focus of much rotavirus research and since 2006, two vaccines have been licensed in many countries around the world.

2.1. Virus Classification

The classical classification system for rotaviruses was derived from their genome composition and the immunological reactivity of three of their structural proteins VP6, VP7 and VP4. Rotaviruses are classified into at least eight groups (A to H) according to the immunological reactivity of the VP6 middle layer protein. Group A rotaviruses are commonly associated with infections in humans. Within group A, two major subgroups (I and II) exist (Iturriza Gomara et al. 2002). The two outer capsid proteins, VP7 and VP4, elicit neutralizing antibodies and are considered to be involved in protection against infection. Using these two proteins a traditional dual classification system of group A rotaviruses into G (depending on VP7 that is a Glycoprotein) and P (from the VP4 that is sensitive to Proteases) types was established (Estes and Kapikian 2007). At least 27 different G-serotypes and 37 P-types have been identified among human and animal rotaviruses, depending on VP7 and VP4, respectively. G serotypes correlate fully with G genotypes as determined by sequence analysis of their VP7 gene. However, many P genotypes do not correlate with P serotypes (Estes and Kapikian 2007). Because VP4 and VP7 are coded by different RNA segments (segment 4 and segments 7–9, respectively), various combinations of G- and P-types can be found in both humans and animals. Viruses

carrying G1P[8], G2[P4], G3[P8] and G4[P8] represent over 90 % of human rotavirus strains co-circulating in most countries, although other G and P combinations are also being isolated in increasing numbers (Iturriza-Gomara et al. 2011). Besides this dual classification system, another system has been utilized including the genotype of the rotavirus enterotoxin, the NSP4 gene (Rodriguez-Diaz et al. 2008; Banyai et al. 2009).

More recently a classification system including the whole genome has been established by the Rotavirus Classification Working Group (RCWG) (Matthijnssens et al. 2008b; Matthijnssens et al. 2011). This classification system allows a better understanding of genomic and antigenic diversity of rotaviruses as well as the reassortment events present in the rotavirus strains. In this genomic classification, each of the 11 RNA segments has been given a tag that includes the traditional G for VP7, P for VP4 protein and E for the NSP4 protein (NSP4 is the viral enterotoxin). The complete genome classification code is as follows: Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx correlating with the VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5/6 encoding genes, respectively (Matthijnssens et al. 2008b; Matthijnssens et al. 2011). Whole genome analysis of rotavirus strains is generating interest in their complete genetic constellations and complex genetic diversity (Matthijnssens and Van Ranst 2012). On the basis of complete group A rotavirus genome sequence comparisons, two major genotype constellations of the non-G, non-P genes; I1-R1-C1-M1-A1-N1-T1-E1-H1 (Wa-like) and I2-R2-C2-M2-A2--N2-T2-E2-H2 (DS-1-like), have been shown to circulate worldwide among humans. Human Wa-like rotavirus A (RVA) strains are believed to have a common ancestor with porcine RVA strains, whereas human DS-1-like strains are believed to have several gene segments which have a common ancestor with bovine RVA strains (Matthijnssens et al. 2008a). A third (minor) human genotype constellation, referred to as AU-1-like (I3-R3-C3-M3-A3-N3-T3-E3-H3), is believed to originate from cats or dogs (Nakagomi et al. 1990).

2.2. Structure of the Virion

The wheel-like structure is the characteristic feature of RV particles under transmission electron microscopy and has the name; “rota” in Latin means wheel. Rotavirus particles are of icosahedral symmetry, consist of three concentric layers of protein and measure ~1000 Å in diameter including the spikes (Estes and Kapikian 2007). The core layer is formed by the VP2 protein entirely surrounding the 11 segments of double-stranded genomic RNA, and proteins VP1 and VP3, which are transcription enzymes attached as a heterodimeric complex to the inside surface of VP2 at the fivefold axis of symmetry (Prasad et al. 1996b) (Fig. 3.3). VP1, the RNA-dependent RNA polymerase, interacts with VP3, the guanyltransferase and methylase protein (Liu et al. 1992). This inner core structure is composed of 120 copies of VP2, which is a RNA-binding protein (Labbe et al. 1991). The addition of VP6 to the VP2 layer produces double-layered particles (DLP). VP6 forms 260 trimers interrupted by 132 aqueous channels of three different kinds in relation

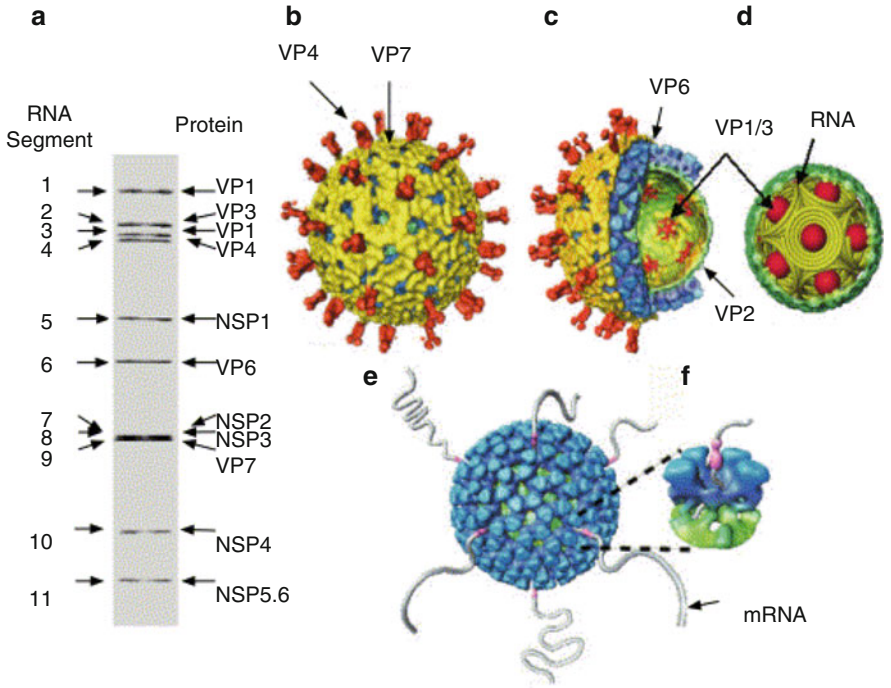

Figure 3.3. Architectural features of rotavirus. (**a**) PAGE gel showing 11 dsRNA segments composing the rotavirus genome. The gene segments are numbered on the *left* and the proteins they encode are indicated on the *right*. (**b**) Cryo-EM reconstruction of the rotavirus triple-layered particle. The spike proteins VP4 are colored in orange and the outermost VP7 layer in yellow. (**c**) A cutaway view of the rotavirus TLP showing the inner VP6 (*blue*) and VP2 (*green*) layers and the transcriptional enzymes (shown in *red*) anchored to the VP2 layer at the fivefold axes. (**d**) Schematic depiction of genome organization in rotavirus. The genome segments are represented as inverted conical spirals surrounding the transcription enzymes (shown as *red balls*) inside the VP2 layer in *green*. (**e** and **f**) Model from cryo-EM reconstruction of transcribing DLPs. The endogenous transcription results in the simultaneous release of the transcribed mRNA from channels located at the fivefold vertex of the icosahedral DLP (Reproduced with permission from Jayaram et al. 2004).

to the capsid's symmetry. The outer capsid of the triple-layered particles (TLP) is composed of two proteins, VP7 and VP4. The smooth surface of the virus is made up of 260 trimers of VP7 and 60 spikes emerging from the viral surface consist of dimers of VP4 (Prasad et al. 1988; Yeager et al. 1990). Rotavirus DLP and TLP contain 132 porous channels which allow the exchange of compounds to the inside of the virus particle. There are 12 type I channels each of which is located at the icosahedral fivefold vertices of the TLP and DLP. Each of the type I channels is surrounded by five type II channels, and 60 type III channels are placed at the hexavalent positions

immediately neighboring the icosahedral threefold axis (Prasad et al. 1988; Yeager et al. 1990).

During cell entry, the TLP loses the VP7 and VP4 proteins, and the resulting DLP becomes transcriptionally active inside the cytoplasm (Estes and Kapikian 2007). VP4 is a non-glycosylated protein of 776 amino acids and has essential functions in the virus cell cycle, including receptor binding, cell penetration, hemagglutinin activity, and permeability of cellular membranes (Estes and Kapikian 2007). VP4 is post-transcriptionally cleaved into the larger VP5* and the smaller VP8* subunits; the cleavage of VP4 enhances virus infectivity by several fold. It has been shown that trypsin cleavage confers icosahedrical ordering on the VP4 spikes, which is essential for the virus to enter the cell (Crawford et al. 2001). Moreover, biochemical studies on recombinant VP4 showed that proteolysis of monomeric VP4 yields dimeric VP5* (Dormitzer et al. 2001). VP7 is a calcium-binding glycoprotein of 326 amino acids with nine variable regions contributing to type-specificity (Nishikawa et al. 1989; Hoshino et al. 1994). In addition, VP7 interacts with integrins αxβ2 and α4β1 (Coulson et al. 1997a; Hewish et al. 2000) and induces polyclonal intestinal B-cell activation during rotavirus infection (Blutt et al. 2004).

When the DLP is located intracellularly, it becomes transcriptionally competent and new mRNA transcripts are translocated from the particle through the type I channels at the fivefold axis (Lawton et al. 1997). Prasad et al. (1988) were the first to propose that these channels in the VP6 layer could be used by mRNA to exit. Cryo-EM studies have confirmed that DLPs maintain their structural integrity during the process of transcription. In a pseudo-atomic model of the T = 13 VP6 layer (Mathieu et al. 2001), a β-hairpin motif of VP6 with a highly conserved sequence that protrudes into the mRNA exit channel may play a functional role in the translocation of the nascent transcripts (Lawton et al. 2000). A detailed mutational analysis of the VP6 layer has contributed to elucidation of determinants of VP6 required for its assembly on VP2 and how VP6 may affect endogenous transcription (Charpilienne et al. 2002).

2.3. The Genome

The genome of rotavirus consists of 11 segments of double-stranded RNA (dsRNA) with conserved 5′ and 3′ ends, ranging from 667 bp (segment 11) to 3302 bp (segment 1) in size. The simian rotavirus strain SA11 totals 6120 kDa and its genes encode for six structural proteins (VP1, VP2, VP3, VP4, VP6, VP7) and six nonstructural proteins (NSP1 to NSP6). The coding assignments, functions and many properties of the proteins encoded by each of the 11 genome segments are now well established (Table 3.4). Protein assignments have been determined by in vitro translation using mRNA or denatured dsRNA and by analyses of reassortant viruses (Estes and Kapikian 2007).

Cryo-EM analysis of rotavirus provided visualization of the structural organization of the viral genome (Prasad et al. 1996b). It was shown that the dsRNA forms a dodecahedral structure in which the RNA double helices,

Table 3.4 Genome segments and proteins of simian rotavirus SA11: coding assignments, functions and biological properties of the encoded proteins (adapted from Estes (2001) and Estes and Kapikian (2007))

Genome segment size (bp)	*ORFs*	*Gene product(s)*	*Protein size aa (Da)*	*Location in virus particle*	*Copy number/ Particle*	*Functions and properties*
1 (3302)	18-3282	VP1	1088 (125,005)	Inner capsid, fivefold axis	12	RNA-dependent RNA polymerase Part of minimal replication comlex Virus specific 3′-mRNA binding Part of virion transcription complex with VP3
2 (2690)	17-2659	VP2	881 (102,431)	Inner capsid	120	Core matrix protein Non-specific ss & dsRNA-binding activity Myristoylated Assembly and RNA-binding activity Part of minimal replication complex Leucine zipper
3 (2591)	50-2554	VP3	835 (98,120)	Inner capsid, fivefold axis	12	Multifunctional capping enzyme: Guanylyltransferase Methyltransferase Located at the vertices of the core Part of virion transcription complex with VP1 Non-specific ssRNA binding
4 (2362)	10-2337	VP4	776 (86,782)	Outer capsid spike	120	VP4 dimers form outer capsid spike Interacts with VP6
	–	VP5*	529 247–776 (60,000)			Virus infectivity enhanced by trypsin cleavage of VP4 into VP5* and VP8* Hemagglutinin Cell attachment protein
	–	VP8*	247 1–247 (28,000)			P-type neutralization antigen VP5* permeabilizes membranes Crystal structure of VP8 fragment (galectin fold) TRAF2 signaling Protection

5 (1611)	31-1515	NSP1	495 (58,654)	Nonstructural	–	Associates with cytoskeleton Extensive sequence diversity between strains Two conserved cysteine-rich zinc-finger motifs Virus specific 5′-mRNA binding Interacts with host IFN regulatory factor 3
6 (1356)	24-1214	VP6	397 (4816)	Middle capsid	780	Major virion protein Middle capsid structural protein Homotrimeric structure Subgroup antigen Myristoylated [15]. Protection (Mechanism?)
7 (1105)	26-970	NSP3	315 (34,600)	Nonstructural	–	Homodimer Virus-specific 3′- mRNA binding Binds eIF4G1 and circularizes mRNA on initiation complex Involved in translational regulation and host shut-off
8 (1059)	47-997	NSP2	317 (36,700)	Nonstructural	–	NTPase activity Helix destabilization activity Non-specific ssRNA-binding Involved in viroplasm formation with NSP5 Functional octamer Binds NSP5 and VP1 Induces NSP5 hyperphosphorylation
9 (1062)	49-1026	VP7	326 (7368)	Outer capsid glycoprotein	780	Outer capsid structural glycoprotein G-type neutralization antigen N-linked high mannose glycosylation and trimming RER transmembrane protein, cleaved signal sequence Ca^{2+} binding Protection

(continued)

Table 3.4 (continued)

Genome segment size (bp)	*ORFs*	*Gene product(s)*	*Protein size aa (Da)*	*Location in virus particle*	*Copy number/ Particle*	*Functions and properties*
10 (751)	41-569	NSP4	175 (20,290)	Nonstructural	–	Enterotoxin Receptor for budding of double-layer particle through ER membrane RER transmembrane glycoprotein Ca++/ Sr++ binding site N-linked high mannose glycosylation Protection Host cell $[Ca^{2+}]_i$ mobilization Pathogen-associated molecular pattern (PAMP)
11 (667)	22-615	NSP5	198 (21,725)	Nonstructural	–	Interacts with VP2, NSP2 and NSP6 Homomultimerizes O-linked glycosylation (Hyper-) Phosphorylated Autocatalytic kinase activity enhanced by NSP2 interaction Non-specific ssRNA binding
	80-355	NSP6	92 (11,012)	Nonstructural	–	Product of second, out-of-frame ORF Interacts with NSP5 Localizes to viroplasm

interacting closely with the VP2 inner layer, are packed around the transcription complexes located at the icosahedral vertices (Fig. 3.3d). VP2 has RNA binding properties and may be responsible for the icosahedral ordering and the closely interacting portions of the RNA. Pesavento et al. (2001) have demonstrated reversible condensation and expansion of the rotavirus genome within the capsid interior under various chemical conditions. This condensation is concentric with respect to the particle center and a dark mass of density is consistently seen in the center of each of the particles (Pesavento et al. 2001). At high pH in the presence of ammonium ions, the genome condenses to a radius of 180 Å from the original radius of 220 Å, and when brought back to physiological pH the genome expands to its original radius (Pesavento et al. 2001). These studies suggest that VP2, through its RNA-binding properties, plays an important role in maintaining the appropriate spacing between the RNA strands in the native expanded state. A plausible and elegant model for the structural organization of the genome that emerges from these studies is that each dsRNA segment is spooled around a transcription enzyme complex at the fivefold axes, inside the innermost capsid layer (Prasad et al. 1996b; Pesavento et al. 2001; Pesavento et al. 2003).

2.4. Mechanisms of Evolution and Strain Diversity

Rotaviruses, like many RNA viruses, display a great degree of diversity. In addition to different G and P types and a variety of combinations of these, there also exists intratypic variation. Three mechanisms, singularly or in combination, are important for the evolution and diversity of rotaviruses, although it is not clear what their relative contributions are to the burden of disease:

(a) *Antigenic drift:* The rate of mutation within rotavirus genes is relatively high because RNA replication is error-prone. The mutation rate has been calculated to be $<5 \times 10^{-5}$ per nucleotide per replication which implies that on average, a rotavirus genome differs from its parental genome by at least one mutation (Blackhall et al. 1996). Point mutations can accumulate and give rise to intratypic variation as identified by the existence of lineages within the VP7 and VP4 genes of particular G and P types. Point mutations can lead to antigenic changes, which may result in the emergence of escape mutants (Palombo et al. 1993; Cunliffe et al. 1997; Maunula and von Bonsdorff 1998; Iturriza Gomara et al. 2002; Estes and Kapikian 2007).

(b) *Antigenic shift:* Shuffling of gene segments through reassortment can occur during dual infection of one cell. Reassortment can, therefore, contribute to the diversity of rotaviruses and there is increasing evidence that reassortment takes place *in vivo* (Ramig 2000). There is also evidence that reassortment, through alterations in protein-protein interactions possibly leading to changes in conformational epitopes, can contribute to the evolution of antigenic types (Chen et al. 1992; Lazdins et al. 1995). Interspecies transmission and the subsequent reassortment have the potential to

enormously increase the diversity of co-circulating rotaviruses. In addition, human rotavirus genes encoding proteins to which the human population is immunologically naïve may allow a rapid spread of the reassortant strain (Iturriza-Gomara et al. 2000). While antigenic drift has always been considered one of the main forces driving rotavirus evolution, extensive analysis of whole genome sequences seems to reveal there are restrictions to this evolution mechanism (Matthijnssens and Van Ranst 2012).

(c) *Gene rearrangements:* The concatemerization or truncation of genome segments and their ORFs has the potential to contribute to the evolution of rotaviruses through the production of new proteins with altered functions (Desselberger 1996).

2.5. Genome Replication

The RNA polymerase activity of DLPs catalyzes the synthesis of the 11 mRNAs of rotaviruses, which range in size from ~0.7 to 3.3 kb. With the exception of gene 11, they are monocistronic (Estes and Kapikian 2007). The nascent transcripts are extruded through channels present at the fivefold-axes of the DLPs (Lawton et al. 1997). The 5′- and 3′-untranslated regions (UTRs) are 9–49 and 17–182 nucleotides in length, respectively. The viral mRNAs serve as templates for the synthesis of minus-strand RNA, to form dsRNA molecules (Chen et al. 1994). The synthesis of dsRNA and the assembly of cores and DLPs occur in viroplasms in the cytoplasm (Estes and Kapikian 2007). There are three species of RNA-containing replication intermediates (RIs) in the infected cells: pre-core RIs, which contain the structural proteins VP1 and VP3; core-RIs with VP1, VP2 and VP3; and double layered RIs, which contain VP1, VP2, VP3 and VP6. The 11 genomic segments are produced and packaged in equimolar amounts in rotavirus-infected cells, which demonstrates that RNA packaging and replication are coordinated processes (Patton and Gallegos 1990). The absence of naked dsRNA in infected cells suggests that packaging takes place before replication (Patton et al. 2003).

The only primary sequences that are conserved among the rotavirus mRNAs are located within the UTRs. Since all 11 mRNAs are replicated by the same VP1-VP2-VP3 polymerase complex, the viral mRNAs almost certainly share common cis-acting signals recognized by the polymerase (Patton et al. 2003). The most remarkable feature of the 3′ end of rotavirus mRNA, as well as that of other members of the *Reoviridae* family, is the absence of a poly(A) tail. Instead, all rotavirus genes and mRNAs end with the same short sequence, UGACC, which is conserved amongst all the segments in group A rotaviruses. Site-specific mutagenesis has revealed that it is the 3′-CC of the 3′ consensus sequence that is most critical for minus-strand synthesis (Chen et al. 2001). In addition, it has been demonstrated that the promoter for minus-strand synthesis is formed by base-pairing in *cis* of complementary sequences proximal to the 5′- and 3′-ends of the viral mRNAs (Chen and Patton 1998). The 3′-consensus sequence also contains a cis-acting signal that acts as a translation enhancer, whose activity is mediated by NSP3 that specifically recognizes the last four to five nucleotides of the 3′-consensus sequence (Poncet

et al. 1994). NSP3 also interacts with the initiation factor, eIF4GI, and facilitates the circularization of viral mRNAs in polysomes, hence increasing the efficiency of viral gene expression (Piron et al. 1998).

The development of a cell-free system that supports the synthesis of dsRNA from exogenous mRNA represents an important milestone in the study of rotavirus replication by providing a tool to analyze the elements in viral mRNAs that promote minus-strand synthesis. This system is based on virion-derived cores which have been disrupted or "opened" by incubation at hypotonic buffer (Chen et al. 1994). Recently, two systems that allow partial reverse genetics on rotavirus have emerged. The first approach utilized a helper virus and neutralizing selection antibodies (Komoto et al. 2006; Komoto et al. 2008). However, in this approach only structural proteins that are able to elicit specific neutralizing antibodies could be modified. In the second approach, the combination of a thermosensitive defect in the NSP2 protein with RNAi-mediated degradation of NSP2 mRNAs allowed the dual selection and recovery of recombinant viruses with modifications in a non-structural protein coding gene (Trask et al. 2010; Navarro et al. 2013).

2.6. Cell Infection

Rotaviruses have a specific tropism *in vivo*, infecting primarily the mature enterocytes of the villi of the small intestine. Several reports suggest that extra-intestinal spread of the virus takes place during infection indicating a wider tropism than previously considered (Blutt et al. 2003; Mossel and Ramig 2003). Rotaviruses can bind to a wide variety of cell lines although only a subset is efficiently infected. These include cells of renal and intestinal origin and transformed cell lines from breast, stomach, lung and bone (Ciarlet et al. 2002b; Lopez and Arias 2004). Most studies on rotavirus replication have been carried out in MA104 cells, which are routinely used to produce progeny virus. However, new investigations into the pathophysiological mechanisms of rotavirus infection are now being performed using in vitro polarized cells like the human intestinal HT-29 and Caco-2 cells (Servin 2003).

Rotaviruses enter into the cell by a complex multistep process in which different domains of the viral surface interact with cell surface molecules that act as receptors (Guerrero et al. 2000a; Guerrero et al. 2000b; Ciarlet and Estes 2001; Ciarlet et al. 2002a; Ciarlet et al. 2002b; Lopez and Arias 2004). Some animal rotavirus strains interact with sialic acid (SA) residues to attach to the cell surface and hence their infectivity is diminished by the treatment of cells with neuraminidase (NA). In contrast, many animal and human strains are NA-resistant (Ciarlet and Estes 1999). The interaction of rotavirus with SA has been shown to depend on the VP4 genotype of the virus and not the species of origin (Ciarlet et al. 2002b). Ganglioside GM3 has been suggested to act as the SA-containing receptor for the porcine rotavirus strain OSU (Rahman et al. 2003) and ganglioside GM1 (NA-resistant) has been identified as the receptor for the NA-resistant human rotavirus strains KUN and MO (Guo et al. 1999a).

The VP8* domain of VP4 is involved in interactions with SA whereas VP5* is implicated in interactions with integrins. The interaction of rotavirus with integrin α2β1 has been shown to be mediated by the DGE integrin-recognition motif, located at amino acids 308–310 of VP4 within VP5* (Zárate et al. 2000). VP4 also contains the tripeptide, IDA, at amino acids 538–540, which is a ligand-binding motif for integrin α4β1 (Coulson et al. 1997); however, the functionality of this site has not been demonstrated. Integrin αvβ3 has also been shown to be involved in the cell entry of several rotavirus strains at a post-attachment step (Graham et al. 2003). Besides, cell-surface heat shock cognate protein, hsc70, has also been implicated as a post-attachment receptor for both NA-sensitive and NA-resistant rotavirus strains (Guerrero et al. 2002). Studies with polarized epithelial cell lines show that the viral entry of SA-dependent strains is restricted to the apical membrane, whereas SA-independent strains enter either apically or basolaterally (Ciarlet et al. 2001). It has been suggested that lipid rafts might play a role in the cell entry of rotavirus (Isa et al. 2004), probably serving as platforms to allow an efficient interaction of cell receptors with the viral particle (Manes et al. 2003; Lopez and Arias 2004).

Recently it has been shown that HBGAs can act as receptors or co-receptors for certain genotypes of the VP4 protein (Hu et al. 2012; Huang et al. 2012; Liu et al. 2012; Liu et al. 2013; Ramani et al. 2013). These findings are relevant from the host genetics point of view because populations with a high prevalence of the Lewis-negative genotype are insensitive to rotaviruses carrying VP4 P[8] genotypes, thereby decreasing the circulation of P[8] genotypes and lowering the efficiency of rotavirus vaccines carrying the P[8] genotype.

Rotavirus infection in polarized, fully-differentiated Caco-2 cells is followed by a defect in brush-border hydrolase expression (Jourdan et al. 1998). Sucrase-isomaltase activity and apical expression are specifically reduced by rotavirus infection without any apparent cell destruction (Jourdan et al. 1998). In addition, viral infection induces an increase in intracellular calcium concentration, damages the microvillar cytoskeleton, and promotes structural and functional injuries at the tight junctions in cell-cell junctional complexes of Caco-2 cells monolayers without damaging their integrity (Brunet et al. 2000; Obert et al. 2000).

2.7. The NSP4 Enterotoxin

The rotavirus nonstructural glycoprotein, NSP4, functions as an intracellular receptor that mediates the acquisition of a transient membrane envelope as sub-viral particles bud into the endoplasmic reticulum. It has been demonstrated that NSP4 binds intracellular DLPs, interacting with VP6 (Estes and Kapikian 2007). Many structural motifs or protein regions have been implicated in the NSP4 biological function. Amino acids 17–20 from the C-terminus are necessary and sufficient for inner capsid particle binding (O'Brien et al. 2000) and the region involved in the retention of the NSP4 protein into the endoplasmic reticulum has been mapped to be between amino acids 85 and 123 at the cytoplasmic region of the protein (Mirazimi et al. 2003). Residues

at positions 48–91, a region which includes a potential cationic amphipathic helix, are involved in membrane destabilization (Tian et al. 1996; Browne et al. 2000). Purified NSP4 or a peptide corresponding to NSP4 residues 114–135 induce diarrhea in young mice after an increase in intracellular calcium levels, suggesting a role for NSP4 in rotavirus pathogenesis (Tian et al. 1994; Ball et al. 1996; Horie et al. 1999; Rodriguez-Diaz et al. 2003). In vitro studies have shown that following rotavirus replication in cells, a functional 7-KDa peptide of NSP4 (amino acids 112–175) is released from virus-infected cells into the medium by a non-classic, Golgi-independent cellular secretory pathway (Zhang et al. 2000). This endogenously produced peptide binds to apical membrane receptors to mobilize intracellular calcium through phospholipase C signaling. Seo et al. (2008) reported that recombinant NSP4 can bind to the metal ion-dependent adhesion site (MIDAS) present on integrins α1β1 and α2β1 and activates intracellular signalling pathways that regulate spreading between cells. Rotavirus and NSP4 alone induce age-dependent diarrhea and chloride permeability changes in mice lacking the cystic fibrosis transductance regulator (CFTR) (Morris et al. 1999). These results indicate that NSP4 is a novel secretory agonist, because the classical secretagogues carbachol and the diterpene forskolin that induce chloride changes by activating cyclic adenosine monophosphate, instead of by mobilizing $[Ca^{2+}]_i$ as a secondary mediator, fail to cause disease in CFTR knockout mice (Morris et al. 1999).

It has been suggested that NSP4 may directly inhibit the functioning of the cellular Na^+-dependent glucose transporter SGLT-1 (Halaihel et al. 2000). In addition, extracellular and/or intracellular NSP4 may contribute to diarrheal pathogenesis by altering the dynamics of intracellular actin distribution and intercellular contacts (Ciarlet and Estes 2001). NSP4 can affect the cytoskeleton in polarized epithelial cells, but how these pleiotropic properties of NSP4 influence its function in morphogenesis or pathogenesis remains unclear (Tafazoli et al. 2001; Zambrano et al. 2012).

It has been demonstrated that NSP4 stimulates the secretion of nitric oxide in cultured epithelial cells as well as *in vivo* (Rodriguez-Diaz et al. 2006; Borghan et al. 2007), indicating its role as an important mediator molecule in rotavirus infection. NSP4 also has adjuvant properties, suggesting a possible role in the innate immune response to rotavirus infection (Kavanagh et al. 2010). More recently it has been reported that NSP4 triggers the secretion of pro-inflammatory cytokines from macrophage-like THP-1 cells and nitric oxide from murine RAW 264.7 cells (Ge et al. 2013). Therefore, NSP4 acts as a pathogen-associated molecular pattern (PAMP) encoded by rotavirus and provides a mechanism for the production of pro-inflammatory cytokines associated with clinical symptoms of infection in humans and animals.

Another relevant finding on the pathophysiology of rotavirus and its enterotoxin has been the elucidation of the mechanism by which rotavirus, or NSP4 alone, activates the secretion of serotonin from enterochromaffin cells in the intestine. It was previously shown that serotonin played a relevant role

in rotavirus diarrhea (Lundgren et al. 2000) and with this new investigation the mechanism of serotonin secretion has been elucidated (Hagbom et al. 2011). Furthermore, a physiological explanation for rotavirus-induced vomiting has also been found (Hagbom et al. 2011).

The significance of immune response to NSP4 in protection against rotavirus infection in humans is still unknown, although it has been shown that NSP4 elicits both humoral and cell-mediated immune responses (Johansen et al. 1999; Ray et al. 2003; Rodriguez-Diaz et al. 2005; Vizzi et al. 2005). The carboxy end of NSP4 is immunodominant (Rodriguez-Diaz et al. 2004) and NSP4 is also able to elicit protective antibodies in the mouse model (Hou et al. 2008), reinforcing the idea that there is a relevant role of NSP4 in immunological protection from rotavirus infection in humans.

3. ASTROVIRUSES

Human astroviruses are non-enveloped viruses with a positive-sense, single stranded, polyadenylated RNA genome between 6.4 and 7.7 kb in length (Matsui 1997). Astroviruses are members of the *Astroviridae* family, divided into two genera: *Mamastrovirus* (MAstVs) and *Avastrovirus* (AAstVs) infecting mammalian and avian species, respectively. So far, astroviruses have been isolated from 31 species of mammals, 14 of them corresponding to different species of bats, which are thought to act as a reservoir (Chu et al. 2008; Zhu et al. 2009; Xiao et al. 2011). The recent discovery of many novel mammalian and avian astroviruses highlights the fact that astrovirus species cannot be established based solely on the host species. For recent update on taxonomic scheme of astroviruses, refer to the ICTV *Astroviridae* Study Group (Bosch et al. 2010).

The "classical" human astroviruses were classified into eight serotypes (HAstV-1 to HAstV-8) according to the antigenic reactivity of the capsid proteins (Lee and Kurtz 1982; Lee and Kurtz 1994; Taylor et al. 2001). Although the two recently identified groups MLB and VA/HMO astroviruses (Finkbeiner et al. 2009a; Finkbeiner et al. 2009b; Kapoor et al. 2009) infect humans, they are extremely genetically divergent from the classical human astroviruses (HAstV). These new strains are genetically closer to animal astroviruses of mink or sheep, than to HAstV, and thus must be considered different *Mamastrovirus* species infecting the same host (Bosch et al. 2010). Phylogenetic characterization of some of these viruses has shown that a single host species may be susceptible to infection by divergent astroviruses. This is the case of the recently described new human astroviruses (HAstV-MLB1 and MLB-2; HMOAstVs A, B, and C, and HAstV-VA1 and VA2) after metagenomic surveillance studies, which were found in patients suffering from gastroenteritis but are genetically unrelated to the classical eight serotypes of humans (HAstV-1 to HAstV-8) (Finkbeiner et al. 2008a;

Finkbeiner et al. 2008b; Finkbeiner et al. 2009a; Finkbeiner et al. 2009b; Kapoor et al. 2009). In fact, the number of astroviruses associated with humans has nearly doubled within the last few years. The term HAstV-VA/HMO has been proposed to refer to these new astroviruses (Bosch et al. 2014). So far, up to four VA/HMO strains have been described (Jiang et al. 2013). The discovery of novel astroviruses in human specimens reveals that there is greater diversity amongst astroviruses than was previously assumed considering the high level of similarity between human astroviruses 1 and 8 (Finkbeiner and Holtz 2013), raising many questions in regard to viral tropism, epidemiology, and potential link to disease.

Astroviruses produce infections mainly in young children, although illness rates increase again in the elderly (Lewis et al. 1989). However, they also cause disease in adults and immunocompromised patients (Cubitt et al. 1999; Coppo et al. 2000). Persistent gastroenteritis in children with no background disease has also been reported, mainly in association with serotype 3 strains (Caballero et al. 2003). Astrovirus infections occur worldwide and their incidence in children with gastroenteritis in both developing and developed countries ranges from 2 to 9 % (Gaggero et al. 1998; Bon et al. 1999; Mustafa et al. 2000; Guix et al. 2002).

Astroviruses are transmitted by the fecal-oral route, and outbreaks have been associated with consumption of sewage-polluted shellfish and ingestion of water from contaminated sources (Pinto et al. 1996; Pinto et al. 2001). They are frequently shed in stools in significant numbers at the onset of illness. In contrast to caliciviruses, astroviruses replicate in vitro in cell lines, which has allowed detailed studies of the viral replication cycle (Matsui 1997; Willcocks et al. 1999; Matsui et al. 2001). Human astroviruses were originally isolated in HEK cells and subsequently adapted to grow in LLCMK2 cells with trypsin-containing media, although without demonstrable cytopathic effects (CPE). The colonic carcinoma cell line, Caco-2, in contrast to LLCMK2 cells, are directly capable of infection with fecally derived astrovirus and display CPE as early as 2 days post-infection (Willcocks et al. 1990; Pinto et al. 1994).

3.1. Structure of the Virion

Astroviruses are generally described to have a diameter of 28 nm (Madeley 1979), although it may vary depending on the source of virus and the method of preparation for EM (Woode et al. 1984). More detailed ultrastructural analysis of human astrovirus serotype 2 grown in LLCMK2 cells in the presence of trypsin revealed particles with icosahedral symmetry and an array of spikes emanating from the surface (Risco et al. 1995). These particles had an external diameter on negatively stained preparations of 41 nm (including spikes). They did not display the star-like surface appearance characteristically found on fecally shed virus. The star-like morphology was inducible after alkaline treatment (pH 10), and further alkalinization (pH 10.5) led to particle disruption. Intact virions generally band at densities of 1.35–1.37 g/ml in CsCl (Caul and Appleton 1982), although banding at densities of 1.39–1.40 have also been

reported (Konno et al. 1982; Midthun et al. 1993). Astroviruses band at a density of 1.32 g/ml in potassium tartrate-glycerol gradient, which preserves the structural integrity of the virus better than CsCl (Ashley and Caul 1982).

3.2. Genome and Proteins

The RNA genomes of several cell culture-adapted human astrovirus strains have been cloned and sequenced (Jiang et al. 1993a; Lewis et al. 1994; Willcocks et al. 1994; Geigenmuller et al. 1997), providing new perspectives on their molecular biology. The organization of the genome includes three long ORFs, designated as ORF1a, ORF1b and ORF2. The length of the three ORFs varies among different viral strains and the largest variation in ORF1a is due to common insertions and deletions (in/del regions) in its 3′ end (Willcocks et al. 1994; Guix et al. 2005). ORF1a (~2700 nt) is located at the 5′ end of the genome and contains transmembrane helices, a 3C-like serine protease motif, a putative protease-dependent cleavage site, and a nuclear localization signal (Jiang et al. 1993a; Willcocks et al. 1994). ORF1b (~1550 nt) contains a RNA-dependent RNA polymerase motif, whereas ORF2 encodes the structural proteins (Matsui et al. 2001).

The nonstructural proteins of the virus are translated from the genomic viral RNA as two polyproteins (Jiang et al. 1993a). One of them contains only ORF1a and the other includes ORF1a/1b, and is translated via a –1 ribosomal frameshifting (RFS) event between ORF1a and ORF1b (Jiang et al. 1993a). Both proteins are proteolytically processed generating a variety of polypeptides, although it is unclear whether the viral protease is responsible for all the cleavages (Geigenmuller et al. 2002a; Geigenmuller et al. 2002b; Kiang and Matsui 2002). An additional ORF (ORF-X) of 91–122 codons, contained within ORF2 in a +1 reading frame, has been described in all HAstVs and some other mammalian viruses (Firth and Atkins 2010). Its initiation codon is located 41–50 nucleotides downstream the ORF2 AUG and might be translated through a leaky scanning mechanism. It remains to be determined whether the putative protein encoded in ORF-X is synthesized in HAstV-infected cells and what is its significance for virus replication (Méndez et al. 2013).

A subgenomic RNA of ~2.8 kb that contains ORF2 is found in abundance in the cytoplasm of astrovirus-infected cultured cells (Monroe et al. 1991; Monroe et al. 1993). The subgenomic RNA (approximately 2400 nucleotides) is translated as a 87 kDa capsid precursor protein that is believed to give rise to three to five smaller, mature capsid proteins in a process that involves trypsin and a putative cellular protease (Monroe et al. 1991; Bass and Qiu 2000; Mendez et al. 2002). The 87 kDa capsid protein is rapidly converted intracellularly to a 79 kDa form, which is found in smaller amounts in the cell supernatants. Bass and Qiu (2000) identified a trypsin cleavage site in a highly conserved region of the ORF2 product (Bass and Qiu 2000). Trypsin-free particles were minimally infectious in Caco-2 cells but became highly infectious after trypsin, but not chymotrypsin, treatment. This trypsin-enhanced infectiv-

ity correlated with conversion of the 79-kDa capsid protein into three smaller peptides of approximately 26, 29 and 34 kDa. However, the apparent molecular weight reported for the smaller mature proteins depends on the astrovirus serotype and whether the virus studied was derived from infected cultured cells or from stools (Matsui et al. 2001).

Astrovirus virus-like particles (VLPs) have been generated by cloning the cDNA corresponding to ORF2 from a human astrovirus serotype 2 virus into a vaccinia virus vector (Dalton et al. 2003). Protein composition of these purified VLPs revealed no substantial difference from that of authentic astrovirus virions when analysed by Western blotting. Trypsin cleavage seemed to be necessary to process the capsid polyprotein into the mature structural proteins. The three virological features which altogether distinguish astroviruses from other positive ssRNA viruses are: the location of the ORF1a and ORF1b encoding regions for non-structural proteins at the 5′ end of the genome and the presence of the ORF2 structural protein encoding region at the 3′ end; the use of a RFS mechanism to translate the RdRp encoded in ORF1b; and the lack of a helicase domain, characteristic of the positive ssRNA viruses with genomes larger than 6 kb. The presence of a VPg protein linked to the 5′-end of the astrovirus genome has also been speculated for many years but its existence has only recently been confirmed for human astroviruses (Fuentes et al. 2012).

4. ENTEROVIRUSES

4.1. Polioviruses

Enteroviruses replicate in the gastrointestinal tract, but the resulting infection is frequently asymptomatic. Symptoms, when they occur, range from paralysis to fever. Enteroviruses, named after the site of replication, rarely cause gastroenteritis. In many cases the enterovirus isolated might merely have been a passenger virus unrelated to the disease (Melnick 2001). The capsids of enteroviruses (family *Picornaviridae*) are composed of four structural proteins (VP1-VP4) arranged in 60 repeating protomeric units with icosahedral symmetry. Among the family members, the capsid proteins are arranged similarly, but the surface architecture varies. These differences account for not only the different serotypes but also the different modes of interaction with cell receptors. The basic building block of the picornavirus capsid is the protomer, which contains one copy of each structural protein. The capsid is formed by VP1 to VP3, and VP4 lies on its inner surface. VP1, VP2, and VP3 have no sequence homology, yet all three proteins have the same topology: they form an eight-stranded antiparallel "β-barrel" core structure (Racaniello 2007). The external loops that connect the beta strands are responsible for differences in the antigenic diversity among the enteroviruses. Neutralization sites are more densely clustered on VP1.

The structures of many human picornaviruses have been resolved and show that they share a number of conserved structural motifs. For example, the capsids of polio- and coxsackieviruses have a groove or canyon surrounding each fivefold axis of symmetry. In contrast, cardioviruses and aphthoviruses do not have canyons (Racaniello 2007). Immediately beneath the canyon floor of each protomer is a hydrophobic pocket occupied by a lipid moiety. These molecules, termed pocket factors, have been shown to stabilize the capsid and their removal from the pocket is a necessary prerequisite to uncoating. The pocket factors have been proposed, from the electron densities and uncoating studies, to be short chain fatty acids (Smyth et al. 2003).

The positive-sense RNA genome is approximately 7.4 kb long and serves as a template for both viral protein translation and viral replication. The 5′ end is covalently linked to a VPg protein. The genome is organized into a long (~740 nucleotide) nontranslated region (5′NTR), which contains the IRES and precedes a single ORF. The ORF is subdivided into three regions, P1 to P3. The P1 region codes for the structural proteins. The P2 and P3 regions encode the nonstructural proteins, essential for viral replication (2A-C and 3A-D). Translation of the ORF gives rise to a single large polyprotein that is posttranslationally modified by virus-encoded proteases. Immediately downstream of the ORF is the 3′ noncoding region (3′NTR; which plays a role in viral RNA replication) and a terminal poly(A) tail (Racaniello 2007).

Human poliovirus, including three serotypes (1–3), is considered a species within the genus *Enterovirus*. A redefinition of the criteria for species demarcation within the genus *Enterovirus* based on molecular and biochemical characteristics has recently been issued by the International Committee on Taxonomy of Viruses (ICTV). Based on these criteria, the genus *Enterovirus* now encompasses 12 species: enteroviruses A through J, and rhinoviruses A through C, according to the Ninth Report of the ICTV (Knowles et al. 2012a). Enteroviruses can cause in humans illnesses ranging from benign upper respiratory infections to severe meningitis and encephalitis. During the summer 2014, an unprecedented disease burden due to enterovirus-D68 (EV-D68) infections was reported from the USA (Midgley et al. 2014).

Poliovirus infections, once responsible for high morbidity and mortality worldwide, are now under control, and their eradication is a priority for the World Health Organization (WHO). Since the Global Polio Eradication Initiative was launched by WHO, the number of polio cases has fallen by over 99 %, from an estimated 350,000 cases in 1988–1919 in 2002. In the same time period, the number of polio-infected countries was reduced from 125 to 7. At present, polio is endemic in three countries: Afghanistan, Nigeria, and Pakistan. Until poliovirus transmission is interrupted in these strongholds, all countries remain at risk, as shown not only by a recent outbreak in Syria, but also in recurrent outbreaks across sub-Saharan Africa (Anonymous 2014). There is a historic opportunity to stop transmission of poliovirus. If the world seizes this opportunity and acts immediately, no child will ever again know the effects of this devastating disease (Dowdle et al. 2001).

4.2. Kobuviruses

Aichivirus, a cytopathic small round virus, was isolated for the first time in 1989 from fecal samples of patients involved in an oyster-associated gastroenteritis outbreak (Yamashita et al. 1991). Since then, several Aichiviruses have been isolated in BS-C-1 cells from patients with gastroenteritis (Yamashita et al. 1995). The virus is commonly found in outbreaks of gastroenteritis in Japan and is often associated with the consumption of oysters (Yamashita et al. 1995). Genetic analyses of Aichivirus revealed that it belongs to the *Picornaviridae* family, but that it is different from any other genus within this family (Yamashita et al. 1998). It was proposed by the ICTV that this virus be assigned to a new genus named *Kobuvirus* (King et al. 1999). "Kobu" means knob in Japanese, which was suggested due to the characteristic morphology of the virion. Currently, the existing species within the genus *Kobuvirus* have been renamed as follows: *Aichi virus* as *Aichivirus A*, *Bovine kobuvirus* as *Aichivirus B* and the porcine kobuvirus as *Aichivirus C* (Knowles et al. 2012b).

Aichivirus can be grown in BS-C-1 and Vero cells, producing CPE characterized by detachment of the cells. By negative staining and electron microscopy the virion shows a rough surface and measures around 30 nm in diameter. Three capsid proteins of 42, 30 and 22 kDa have been described (Yamashita et al. 1998). Aichi virus possesses a genome of single-stranded RNA of 8280 nucleotides, excluding a poly(A) tract. It contains a large ORF with 7299 nucleotides that encodes a polyprotein precursor of 2432 amino acids. The ORF is preceded by 744 nucleotides and followed by 237 nucleotides and a poly(A) tail. The precise secondary structure of the 5′ non-translated region (5′-NTR) has not been defined although an IRES similar to that of other members of the *Picornaviridae* family has been reported (Sasaki et al. 2001). The complete nucleotide sequence of Aichivirus (GenBank accession no. AB010145) has been determined (Yamashita et al. 1998).

Aichiviruses (AiVs) are subdivided into three genotypes (A, B, and C) based on differences in the nucleotide sequences at the 3C–3D junction (Yamashita et al. 2000; Ambert-Balay et al. 2008). Yamashita et al. (1998) showed that the VP0 capsid protein is expressed in the mature AiV particles and does not undergo cleavage (Yamashita et al. 1998), in contrast to other picornaviruses. Also the 2A and leader (L) proteins of Aichivirus have no protease or autocatalytic motifs as documented for other picornaviruses and their function remains unknown (Yamashita et al. 1998).

The organization of the deduced amino acid sequence of the polyprotein encoded by the Aichivirus genome is analogous to that of the other picornaviruses. Preceded by the L protein, there is a P1 region corresponding to the structural proteins (VP0, VP3 and VP1, with molecular weights of 42, 30 and 22 kDa, respectively), followed by the P2 and P3 regions which contain sequences encoding nonstructural proteins (2A-C, 3A-D). The 2A protein of Aichivirus contains conserved motifs that are characteristic of the H-rev107 family of cellular proteins involved in the control of cellular proliferation (Hughes and Stanway 2000). Amino acid sequences of the 2C, 3C and 3D

regions are well aligned with the corresponding sequences of other picornaviruses. The 3B protein corresponds to the VPg protein and the 3C protein is the protease which contains conserved motifs characteristic of all picornaviruses (Yamashita and Sakae 2003). The relationship of kobuviruses to other picornaviruses has been analysed based on the 3D amino acid sequence of the RNA polymerase within the polyprotein sequence (Hughes 2004).

Recently, a structurally distinct IRES was described (a fifth class of IRES) in members of *Kobuvirus,* as well as in *Salivirus* ("stool Aichi-like" virus) and *Paraturdivirus*, new genera of the *Picornaviridae* (Sweeney et al. 2012). Kobuviruses associated with infections in cattle and swine have been isolated and characterized (Yamashita et al. 2003; Reuter et al. 2009). By next generation sequencing, canine kobuviruses have also been detected in dogs in the USA (Kapoor et al. 2011; Li et al. 2011) and Europe (Carmona-Vicente et al. 2013). Evidence for a role of the virus in disease is lacking, although high seroprevalence in both dogs and cats suggests that infection is common (Carmona-Vicente et al. 2013). Other proposed kobuvirus species are mouse kobuvirus (MKoV) and sheep kobuvirus (SKV) (Reuter et al. 2011).

5. HEPATITIS A VIRUS

Hepatitis A virus (HAV), the prototype of the genus *Hepatovirus* in the family *Picornaviridae* (Minor 1991), is a hepatotropic virus which represents a significant problem for human health (Hollinger and Emerson 2001). The infection is mainly propagated via the fecal-oral route, and although transmission remains primarily from person-to-person, waterborne and foodborne outbreaks of the disease have been reported (De Serres et al. 1999; Hutin et al. 1999; Fiore 2004). The HAV was originally classified as enterovirus type 72 because its biophysical characteristics are similar to those of enteroviruses. However, later studies demonstrated that HAV nucleotide and amino acid sequences are different from those of other picornaviruses, as are the predicted sizes of several HAV proteins (Cohen et al. 1987b). This virus is difficult to cultivate in cell cultures and usually replicates very slowly without producing CPE, which has hampered its characterization. The virus is resistant to temperatures and drugs that inactivate other picornaviruses and is stable at pH 1. There is only a single serotype of human HAV with one immunodominant neutralization site (Lemon and Binn 1983), which is composed of closely clustered epitopes defined by two major groups of escape mutants that include residues 70, 71 and 74 of VP3 and residues 102, 171 and 176 of VP1 (Nainan et al. 1992; Ping and Lemon 1992). A second epitope is the glycophorin A binding site, represented by mutants around residue 221 of VP1 (Ping and Lemon 1992; Sanchez et al. 2004). Finally, there is a third and still undefined epitope, represented by escape mutants to the 4E7 monoclonal antibody (mAb) (Pinto et al. 2012).

A significant degree of nucleic acid variability has been observed among different isolates from different regions of the world (Robertson et al. 1992; Costa-Mattioli et al. 2001). The molecular basis of this genetic variability may be the high error rate of the viral RNA-dependent RNA polymerase and the absence of proofreading mechanisms (Sanchez et al. 2003). The HAV has a buoyant density of 1.32–1.34 g/cm^3 in CsCl and sediments with 156S during sucrose gradient centrifugation (Coulepis et al. 1982). Infectious viral particles with higher (1.44 g/cm^3) as well as lower (1.27 g/cm^3) buoyant densities have also been reported (Lemon et al. 1985).

Phylogenetic analysis based on a 168-base segment encompassing the VP1/2A junction region of the HAV genome has established the classification of human and simian isolates into six different genotypes (I-VI); genotypes I, II, III include human isolates (Robertson et al. 1992). About 80 % of the human isolates belong to genotype I, which has been subdivided into two subgenotypes, IA and IB (Fujiwara et al. 2003). This classification is based on a 168 nucleotide sequence at the VP1/2A junction (Robertson et al. 1992). Genotypes I, II and III contain subgenotypes defined by a nucleotide divergence of 7–7.5 %. Genetic diversity of HAV is evidenced by the emergence of new subgenotypes (Perez-Sautu et al. 2011).

5.1. The Genome

The genome is linear, single-stranded RNA of messenger sense polarity, approximately 7.5 kb in length, and a capsid containing multiple copies of three or four proteins named VP1, VP2, VP3 and a putative VP4 encoded in the P1 region of the genome (Racaniello 2007). The presence of this fourth protein, VP4, has been described repeatedly, but its apparent molecular weight (7–14 kDa) that has been reported does not correspond to those predicted from nucleic acid sequence data (1.5 or 2.3 kDa) (Weitz and Siegl 1998). The P2 and P3 regions encode nonstructural proteins associated with replication.

Detailed analysis of the HAV genome has been accomplished with cloned or RT-PCR–amplified cDNA. Cultured cells can be infected with RNA transcribed from cloned HAV cDNA (Cohen et al. 1987a). The genome differs from that of *Caliciviridae* in that the genes encoding the nonstructural and structural proteins are located at the 3′ end and 5′ end, respectively (Weitz and Siegl 1998). The HAV genome is divided into a 5′ nontranslated region (5′NTR) of 735 nucleotides, a long open reading frame of 6681 nucleotides encoding a polyprotein of 2227 amino acids, and a 3′ nontranslated region (3′NTR) 63 nucleotides in length.

The 5′NTR contains a cis-acting IRES, which initiates cap-independent translation directed to a particular AUG triplet several hundreds of nucleotides downstream (Glass et al. 1993; Brown et al. 1994; Ali et al. 2001; Borman et al. 2001; Kang and Funkhouser 2002). It has been demonstrated that the IRES is located between nucleotides 151 and 734, which is able to direct internal initiation of translation in a cap-independent manner (Brown et al. 1994).

However, a cap-dependent message effectively competes with the IRES of HAV (Glass et al. 1993). Translational efficiency of this IRES may be dependent on the availability of intact cellular proteins such as the p220 subunit of the eukaryotic initiation factor eIF-4 F, and requires the association between the cap-binding translation initiation factor (eIF4E) and eIF4G (Ali et al. 2001; Borman et al. 2001). The 5′-NTR is the most highly conserved region in the HAV genome among all strains sequenced to date, with a 95 % nucleotide identity. By contrast, the 3′-NTR region shows the highest (up to 20 %) degree of variability. The presence of a short cis-acting element has been described; it is 23 nucleotides long and specifically interacts with proteins involved in the establishment and maintenance of the persistent type of infection characteristic of most HAV isolates. Several unidentified cytoplasmic and ribosomal proteins in infected cells bind to the 3′ end of HAV RNA, indicating an intimate and dynamic interaction between host proteins and viral RNA (Kusov et al. 1996). The poly(A) tail is also involved in the formation of RNA/protein complexes.

Analogous to other picornaviruses, the coding region can be subdivided into P1, P2 and P3 subregions, which specify proteins 1A-D, 2A-C, and 3A-D, respectively. The polyprotein is further processed into the four structural and seven nonstructural proteins by proteinases encoded in and around the 3C region (Probst et al. 1998). Proteins 1A to 1D correspond to the structural proteins VP1-VP4. As in other picornaviruses, the 5′ end of HAV genome is covalently linked to VPg protein, which is specified by 3B, instead of the classical m7G cap structure (Weitz et al. 1986). The 2C gene carries a guanidine resistance marker and is assumed to play a role in viral RNA replication (Cohen et al. 1987b). The 3D region is the RNA-dependent RNA polymerase, which shows the highest degree of homology (29 %) with the corresponding sequence in the poliovirus type 1 protein (Cohen et al. 1987b). A region of 18 amino acids considered to be essential for an active polymerase is present in the 3D region. This sequence contains a conserved motif of two aspartate residues flanked by hydrophobic amino acids that might function as a GTP-binding domain (Kamer and Argos 1984). Replication efficiency seems to be controlled by amino acid substitutions in the 2B and 2C regions (Yokosuka 2000).

It has been proposed that HAV presents a higher codon usage bias compared with other members of the *Picornaviridae* family, which is characterized by the adaptation to use abundant and rare codons (Sanchez et al. 2003). A consequence of this special codon bias is an increase in the number of rare codons used. The critical role of HAV codon usage, and particularly of these clusters of rare codons of the capsid-coding region, has been shown in functional genomic studies during the process of adaptation of HAV to conditions of artificially induced cellular shut-off (replication in the presence of actinomycin D) (Aragones et al. 2010). It has also been suggested that this codon usage contributes to the low antigenic variability observed within the HAV capsid (Aragones et al. 2008). A total of 15 % of the surface capsid residues are

encoded by rare codons. These rare codons are highly conserved among the different HAV strains (Sanchez et al. 2003) and their substitution is negatively selected even under specific immune pressure (Aragones et al. 2008).

5.2. Proteins

The genomes of all picornaviruses encode a single polyprotein which is co- and post-translationally cleaved by virus-encoded proteinase(s). In contrast to well-characterized proteolytic events in the polyproteins of viruses within the genera *Enterovirus* and *Rhinovirus*, this processing has been difficult to characterize in the genus *Hepatovirus*. It has been shown that the primary cleavage of the HAV polyprotein occurs at the 2A/2B junction, which has been mapped by the N-terminal sequencing of 2B. This primary cleavage is mediated by the $3C^{pro}$ proteinase, the only proteinase known to be encoded by the virus (Martin et al. 1995; Gosert et al. 1996). The P1-2A capsid protein precursor is probably released from the nonstructural protein precursor (P3-2BC) as soon as $3C^{pro}$ is synthesized, as the full-length polyprotein has not been observed in these studies. A P1-2A precursor produced in a cell-free translation system has been shown to be cleaved in vitro by recombinant $3C^{pro}$ to generate VP0 (VP4-VP2), VP3 and VP1-2A (Malcolm et al. 1992). This VP1-2A polypeptide is unique to the hepatoviruses; it associates with VP0 and VP3 to form pentamers, intermediates in the morphogenesis of HAV particles (Borovec and Andersen 1993). The mature capsid protein VP1 is subsequently derived from the VP1-2A precursor later in the morphogenesis process. It has been hypothesized that the maturation of VP1 is dependent on $3C^{pro}$ processing of the VP1-2A precursor (Probst et al. 1998). However, it has also been shown, using recombinant vaccinia viruses expressing relevant HAV substrates, that $3C^{pro}$ is incapable of directing the cleavage of VP1 from the VP1-2A precursor. Therefore, maturation of VP1 could not depend on processing by $3C^{pro}$ proteinase (Martin et al. 1999).

5.3. Virus Replication

The main target cells for HAV replication are hepatocytes, although HAV antigen has also been detected in crypt cells of the intestine and Kupffer cells of the liver (Asher et al. 1995). Two cellular receptors for HAV have been proposed: the HAV cell receptor 1 (HAVCR1), which belongs to the T-cell immunoglobulin mucin family (Kaplan et al. 1996) and the asialoglycoprotein receptor, which binds and internalizes IgA-coated HAV complexes (Dotzauer et al. 2000). The general scheme of the replicative cycle of HAV is very similar to that of other picornaviruses (Racaniello 2007). After interaction with the receptor(s), the uncoating of the positive-sense RNA viral genome takes place. This process is extremely slow in HAV (at least in vitro with cell-adapted strains) taking several hours compared with the 30-min period common to most picornaviruses (Bishop and Anderson 2000). The HAV typically has a protracted and non-cytolytic replication cycle in cell culture and fails to shut down host cell metabolism (Lemon and Robertson

1993). Even after successful adaptation to growth in cell cultures, replication of HAV is a slow process that terminates in a state of persistent infection (de Chastonay and Siegl 1987). Cytopathic HAV strains have been recovered only from persistently infected cell cultures. Maximal levels of viral RNA synthesis can be detected at 24 h after infection and exponential production of progeny virus continues up to day 4 post-infection. Lysis of infected cells may become apparent within 3–9 days post-infection and yield of progeny virus rarely exceeds 10^7 $TCID_{50}$/ml (Siegl et al. 1984). Cap-independent translation of the viral genome takes place through the IRES within the 5′ noncoding region. The polyprotein is co- and post-translationally processed by the viral protease and the newly synthesized RNA-dependent RNA polymerase, as well as several membrane-interacting proteins, assemble at the 3′ end of the genomic RNA to start the synthesis of a negative-strand copy of the viral genome. Finally, after the structural proteins are assembled into capsid particles and the positive-strand RNA molecules are packaged, the newly synthesized virions are secreted across the apical membrane of the hepatocyte (Pinto et al. 2012).

6. HEPATITIS E VIRUS

The hepatitis E virus (HEV) is a nonenveloped, hepatotropic virus transmitted via the fecal-oral route or through uncooked animal products and contaminated water. It is classified as a *Hepevirus* in the family *Hepeviridae* (Emerson and Purcell 2007). Of the four known genotypes, genotype 3 is responsible for autochthonous infections in industrialized countries, with a seroprevalence in some European countries estimated as high as 22 %. Most of the HEV infections are caused by genotype 1 or 2 and occur in high-prevalence areas in East and South Asia where sanitary conditions are poor. Large outbreaks of HEV have also been reported from these areas, as from other areas of overcrowding and poor sanitation such as refugee camps (Ahmed et al. 2013). Chronic hepatitis E caused by HEV genotype 3 has been observed in immunosuppressed patients especially transplant recipients (Buffet et al. 1996). Serology is not sufficiently sensitive, especially in immunosuppressed patients, making PCR identification the preferred test for diagnosing active infection (Narayanan et al. 2005; Candido et al. 2012).

6.1. The Genome

The HEV contains a ~7.2-kb, single-stranded, positive sense, 5′-capped RNA genome. It consists of short 5′- and 3′ UTRs flanking three partially overlapping ORFs, namely, ORF1, ORF2, and ORF3 (Tam et al. 1991). The UTRs and a conserved 58-nucleotide region within ORF1 are likely to fold into conserved stem-loop and hairpin structures. These structures and a sequence closer to the 3′ end of ORF1, which has homology to the alphavirus junction

region, are proposed to be important for RNA replication (Purdy et al. 1993). The region between the end of ORF1 and start of ORF3/ORF2 appears to be complex and contains regulatory elements (Ahmad et al. 2011).

The cloning and sequencing of the HEV genome was first reported from cDNA libraries made from the bile of macaques experimentally infected with stool samples obtained from HEV patients (Reyes et al. 1990; Tam et al. 1991). Whole genomic sequences from various genotypes and different geographical isolates of HEV have also become available (Huang et al. 1992; Panda et al. 2000). Three RNA species of around 7.2, 3.7, and 2 kb, designated as the genomic and two subgenomic RNAs, respectively, were detected in the liver tissue of macaques experimentally infected with HEV (Tam et al. 1991). In this model, the 7.2 kb RNA was proposed to be translated into the ORF1 protein and the 3.7 and 2 kb subgenomic RNAs into ORF3 and ORF2 proteins, respectively (Ahmad et al. 2011). However, in stable Huh-7 cell lines made from functional HEV RNA replicons expressing the neomycin resistance gene from ORF2 and ORF3, only the genomic RNA and one subgenomic RNA were observed (Graff et al. 2006). This model was confirmed by intrahepatic inoculation of wild type and mutant genotype 3 swine HEV replicons into pigs (Huang et al. 2007). Mutation of AUG1 or the insertion of a T base, as in genotype 4, did not affect virus infectivity or rescue, but the mutation of AUG3 abolished virus infectivity.

Support for a single subgenomic RNA model also comes from PLC/PRF/5 cells that were either transfected with infectious genotype 3 viral RNA produced in vitro from a cloned cDNA or inoculated with fecal material containing genotype 4 HEV. The RNA isolated from these cells showed only the 2.2 kb subgenomic species, whose 5′ end mapped to nucleotide 5122 (Ichiyama et al. 2009). The viral negative-strand RNA is proposed to be a template for the synthesis of the positive-strand genomic and subgenomic RNAs, the latter from within the junction region in a primer-independent manner (Ahmad et al. 2011). The junction region of the negative-strand RNA is predicted to fold into a stable stem-loop structure and to play important roles in HEV replication (Cao et al. 2010).

6.2. Genetic Variants

By comparison of the full-genomes of HEV isolates, four major genotypes and several subtypes within each genotype have been identified (Lu et al. 2005). An avian HEV, initially considered as HEV genotype 5, is now proposed as a new species within the family *Hepeviridae* (Meng et al. 2011). A unique strain of HEV was identified from farmed rabbits in China, which shared 74–79 % nucleotide sequence identity to existing HEV strains and 46 % identity to avian HEV (Zhao et al. 2009). Recently, HEV-like viruses were also isolated and characterized from Norway rats (Johne et al. 2010), wild boars (Takahashi et al. 2011) and ferrets (Raj et al. 2012). The rabbit HEV appears to be a distant variant of genotype 3. Interestingly, a virus

isolated from cutthroat trout in California, but not associated with disease, bears only 18–27 % sequence similarity to avian or mammalian HEVs and was proposed as a new genus within the family *Hepeviridae* (Batts et al. 2011).

6.3. Proteins

The ORF1 nonstructural polyprotein of HEV is 1693 amino acids (~180 kDa) and contains several functional motifs and domains present in the nonstructural proteins of other positive-stranded RNA viruses. The ORF1 encodes a nonstructural polyprotein with four predicted functional domains, designated as methyltransferase (MeT), papain-like cysteine protease (PCP), helicase (Hel) and RNA-dependent RNA polymerase (RdRp). Besides these regions, two other regions designated X (and also called the macro domain) and Y, share significant homology with nonstructural proteins of other positive-strand RNA viruses. A polyproline region (PRP) or hypervariable region upstream of the macro domain may act as a flexible hinge. It is not entirely clear whether the ORF1 polyprotein is processed into biochemically distinct units, as is the case with other positive-strand RNA viruses. When expressed in mammalian cells using recombinant vaccinia viruses, ORF1 yielded processed products of 78 and 107 kDa (Ropp et al. 2000), whereas its expression in *E. coli* or HepG2 hepatoma cells showed no processing (Ansari et al. 2000).

Macro domain proteins hydrolyze ADP-ribose 1"-phosphate (ADPR-1"P), a product of cellular pre-tRNA splicing associated with poly(ADP-ribose) polymerase-1 (PARP-1), suggesting some role in cellular apoptosis (Egloff et al. 2006). It has also been suggested that the viral macro domains could have a role in the viral RNA replication and/or transcription. Since the HEV macro domains can bind poly(ADP-ribose) in the presence of poly(A), they could recruit poly(ADP-ribose)-modified cellular factors to the replication complex while bound to viral polyadenylated RNA (Neuvonen and Ahola 2009).

The ORF2 protein is the viral capsid protein; the crystal structure of a truncated recombinant ORF2 protein has been elucidated, but the size of the protein in mature virions remains unknown. The full-length protein is composed of 660 amino acid residues. The capsid protein contains three linear domains: S, M, and P (Guu et al. 2009; Yamashita et al. 2009; Xing et al. 2010). Variations in the ORF2 domains could influence cellular or humoral immune responses. The M domain contains T cell epitopes (Aggarwal et al. 2007). It is also a potential receptor binding site, as it contains a sequence that is strictly conserved among all genotypes (Guu et al. 2009). The P domain forms dimeric spikes on the surface of the capsid (Meng et al. 2001; Yamashita et al. 2009) and contains neutralization epitopes (Riddell et al. 2000).

When expressed in animal cells in culture, non-glycosylated and glycosylated ORF2 proteins of 74 and 88 kDa were observed (Zafrullah et al. 1999; Graff et al. 2008). A genotype 3 ORF2 protein expressed using recombinant vaccinia virus was also glycosylated and localized to the ER, Golgi, and surface of infected cells (Jimenez de Oya et al. 2012). A truncated protein of 56 kDa

(amino acids 112–607) can self-assemble in insect cells to form virus-like particles of 23–24 nm in diameter (Li et al. 1997). The ORF2 protein localizes to the ER, and some of the protein is retrotranslocated to the cytoplasm through an ER-associated degradation pathway (Surjit et al. 2007). It has also been demonstrated that ORF2 activates the phosphorylation of the eukaryotic initiation factor 2a (eIF2a), an increased expression of the ATF-4 transcription factor, and activation of the pro-apoptotic gene CHOP (John et al. 2011). An infectious cDNA clone of HEV has been constructed that propagates efficiently in cultured PLC/PRF/5 cells (Yamada et al. 2009). Using this model the intracellular expression and secretion of an 83 kDa ORF2 protein was shown. Though these authors did not directly test glycosylation of the ORF2 protein, a size significantly larger than the predicted size of ~72 kDa, suggests this possibility (Ahmad et al. 2011).

Another controversial issue is the existence of two types of virions: non-enveloped virions found in fecal samples, and "enveloped" virions found in serum samples (Takahashi et al. 2008; Yamada et al. 2009). It was reported that the enveloped virus is associated with the ORF3 protein and lipids, but the structure is largely unclear. Further studies are required to evaluate this subject (Ahmad et al. 2011). The ORF3 protein is a small, phosphorylated protein of 113 or 114 amino acids, whose function(s) has not been fully defined. It is dispensable for replication of HEV in vitro in cell lines (Emerson et al. 2006), but is required for infection in the macaque model of experimental infection (Graff et al. 2005). This suggests that the ORF3 protein functions as a viral accessory protein probably affecting the host response. The ability of the ORF3 protein to interact with multiple cellular proteins suggests its potential role in optimizing the cellular environment for viral infection and replication. ORF3 is a versatile, multifunctional protein that activates the extracellular regulated kinase (Erk), a member of the MAPK family, and this depends upon its ability to bind and inactivate an Erk-specific MAPK phosphatase (Kar-Roy et al. 2004).

6.4. Replication

The primary site of HEV replication is the liver, with hepatocytes being the most likely cell type. However, non-hepatic cell lines such as A549 lung carcinoma cells or Caco-2 colon carcinoma cells also support in vitro replication of HEV. No cellular receptor for HEV has been identified yet. It has been suggested that HEV enters liver cells through receptor-dependent clathrin-mediated endocytosis, although other entry pathways are also feasible (Kapur et al. 2012). Hsp90 and tubulin appear to be involved in capsid protein intracellular trafficking (Zheng et al. 2010). The virus uncoats to release the viral RNA that is translated in the cytoplasm into nonstructural proteins including the RNA-dependent RNA polymerase that replicates the positive sense genomic RNA into negative sense transcripts. The latter then act as template for the synthesis of 2.2 kb subgenomic RNA as well as full-length positive viral genomes (Graff et al. 2006).

The ORF2 protein packages the genomic positive sense RNA into progeny virions. Probably the ORF3 protein, together with lipids, coats the viral particle during the budding process (Takahashi et al. 2008). Although HEV is a non-enveloped virus, its association with lipids, the subcellular localization of the ORF3 protein to endosomes (Chandra et al. 2008), and a requirement for its PSAP motif in the viral egress (Nagashima et al. 2011), suggests that HEV follows the vacuolar protein sorting (VPS) pathway for its release from infected cells. The molecular virology of HEV will become better understood with the development of replicon- and infection-based in vitro cell culture models.

7. ENTERIC ADENOVIRUSES

Adenoviruses are non-enveloped, icosahedral viruses with a diameter of 70–90 nm. Five genera are currently recognized within the *Adenoviridae* family: two genera (*Mastadenovirus* and *Aviadenovirus*) that have probably co-evolved with mammals and birds, two genera with a broader range of hosts (*Atadenovirus* and *Siadenovirus*) and *Ichtadenoviruses* infecting fish (Harrach et al. 2011). The adenoviruses are species-specific and generally replicate only in cells derived from their native host. Human adenoviruses are associated with a variety of infectious diseases affecting the respiratory, urinary and the gastrointestinal tracts and the eyes (Horwitz 2001). To date, 51 serotypes of human adenoviruses have been recognized, which are classified into six subgroups (A to F) based on immunological properties, oncogenicity in rodents, DNA homologies, and morphological properties (Harrach et al. 2011). Adenoviruses have a buoyant density in cesium chloride of 1.33–1.34 g/cm^3. The capsid is composed of 252 capsomeres, of which 240 are hexons and 12 are pentons. Inside the capsid is a single molecule of linear, double-stranded DNA (Shenk 2001).

The enteric adenoviruses were originally identified from stool samples of infants with acute gastroenteritis (Flewett et al. 1973b) and have been consistently associated with gastroenteritis in children through epidemiological and clinical studies (Uhnoo et al. 1983; Uhnoo et al. 1984). The enteric adenoviruses are responsible for 5–20 % of cases of acute diarrhoea in children (Uhnoo et al. 1984; Kotloff et al. 1989; Uhnoo et al. 1990; Bon et al. 1999) and are found in clinical samples throughout the year with little seasonal variation (de Jong et al. 1983).

Enteric serotypes 40 and 41 have been designated as subgroup F adenoviruses. They share the adenovirus group antigen and are distinguished from each other and from other non-enteric serotypes by serology and DNA restriction patterns (Wadell 1984). The enteric serotypes are shed in large numbers from the gut of infected patients and were originally described as being non-cultivable or 'fastidious' adenoviruses because they could not be cultivated in

routine cell cultures that generally supported the propagation of other adenovirus types. However, it was found that these viruses could be propagated in Graham 293 cells, a cell line of human embryonic kidney (HEK) transformed with adenovirus 5 early (E) region 1 (Graham et al. 1977; Takiff et al. 1981), although at lower levels than other serotypes. This suggests that E1 functions are poorly expressed and therefore it was postulated that the inability of adenovirus types 40 and 41 to grow on cell lines normally supportive for other adenovirus types was due to the relative inability of the adenovirus 41 E1A gene to transactivate other adenovirus 41 genes (Takiff et al. 1984; Van Loon et al. 1985a). The Graham 293 cell retains the E1A and E1B regions of the adenovirus genome covalently linked to the host DNA. The mechanism of facilitation of the growth of the EAd40 in 293 cells seems to be a function of the E1B-55 kDa protein (Mautner et al. 1989). Grabow et al. (1992) reported another cell line (PLC/PRF/5 or primary liver carcinoma cells) also supported an efficient propagation of laboratory strains of adenovirus types 40 and 41. Efficient replication of adenovirus types 40 and 41 has also been achieved in other cell lines, like Hep-2 cells, Chang conjunctiva cells and Caco-2 cells (Perron-Henry et al. 1988; Pinto et al. 1994).

The adenovirus 40 genome has been sequenced (Davidson et al. 1993) (GenBank accession. no. L19443) and described in detail (Mautner et al. 1995). The main differences between adenovirus 40 and the other human adenovirus serotypes are the presence of two distinct fibre genes, a single VA gene involved in late translation, and a highly divergent E3 region. The adenovirus 41 growth restrictions in cell cultures seem to be less severe than those of serotype 40, as a number of cell lines support the propagation of serotype 41 and not adenovirus 40 (de Jong et al. 1983; Uhnoo et al. 1983; van Loon et al. 1985b). The adenovirus 41 blockade in replication occurs within the early phase of the infectious cycle (Tiemessen et al. 1996). The ability of adenovirus 40 E1A encoded products (proteins 249R and 221R) for *trans*-activation has also been investigated, (van Loon et al. 1987; Ishino et al. 1988); it has been found that the adenovirus 40 E1A promoter does indeed contain transcription factor binding sites sufficient for *trans*-activation by the adenovirus 5 E1A 289R protein. It is possible that adenovirus 40 has evolved to use components of the RNA processing machinery that are unique to enterocytes (Stevenson and Mautner 2003).

It has been suggested that interferon (IFN) sensitivity could at least in part be responsible for the fastidious growth of species F human adenoviruses. Experiments in conjunctival cells suggest that these viruses are defective in their ability to circumvent the antiviral actions induced by IFN (Tiemessen and Kidd 1993). Highly differentiated Caco2 cells were used as a model to determine whether HAdV-40 is sensitive to the effects of type I and type II IFNs. Infection counts showed that HAdV-40 infection rates were reduced significantly when the cells were pre-treated with IFN-α, compared with those either mock-treated or treated with IFN following infection. Species F adenoviruses may have adapted to tissues that are restricted in their ability to mount

an inflammatory cytokine response (Sherwood et al. 2012). Hence, the use of intestinal cell cultures may lead to a better understanding of adenovirus 40 replication and pathogenicity.

8. SUMMARY

Foodborne and waterborne viruses cause acute gastroenteritis (caliciviruses–noroviruses and sapoviruses, rotaviruses, astroviruses, and enteric adenoviruses), hepatitis (hepatitis A virus and hepatitis E virus), and other diseases. Other enteric viruses like kobuviruses, coronaviruses, toroviruses, and picobirnaviruses also may cause diarrhea, although the causative role for some of these viruses in humans is controversial. In addition, next-generation sequencing technologies have allowed the discovery of new enteric viruses (novel astroviruses, kobuviruses, saliviruses, etc.). Human caliciviruses have been recognized as the leading cause of acute gastroenteritis outbreaks and sporadic cases in children and adults worldwide. Enteric caliciviruses belonging to the *Norovirus* and *Sapovirus* genera still remain refractory to routine cell culture propagation. This limitation has hampered our ability to investigate their biology, pathogenesis, and host immunity, although molecular approaches have provided new insights into these areas. The morphology, composition, and structure of several enteric viruses have been elucidated. Cryo-electron microscopy and x-ray crystallography have been crucial for this purpose. Biochemical and structural studies of virus-like particles produced by recombinant baculoviruses are contributing to a better understanding of the structure-function relationships of the capsid proteins. Viral genome organization is being clarified for all of these viruses, as well as their replication and gene expression strategies. Most of the proteins encoded by the viral genomes have been characterized and their functions identified. Sequence analyses of viral genes have been applied in molecular epidemiology and taxonomy studies. However, many questions still remain to be answered.

Reverse genetics and replicon systems have provided an important tool for the study of replication and gene expression of different enteric viruses. The MNV constitutes an excellent model to analyze HuNoV replication cycle in the laboratory. Biochemical characterization of viral interactions with cells and analysis of the functional properties of the viral proteins are providing a better understanding of the pathogenesis of enteric viruses. Rotavirus NSP4 was the first viral enterotoxin to be characterized. Several cell membrane molecules have been identified recently as being receptors or attachment ligands for different enteric viruses (i.e., integrins and hsc70 for rotaviruses, HBGA carbohydrates for noroviruses). Studies on human susceptibility to norovirus infections have characterized some resistant, non-secretor (Se-) individuals in the population, which is a breakthrough in our knowledge of norovirus-host interactions. Similarly, molecular analyses of orally transmitted viruses causing hepatitis are clarifying the phylogenetic relationships between these viruses and other viral genera, as well as their pathophysiological mechanisms.

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