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Abstract

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INACTIVATION OF PICORNAVIRUSES AND CALICIVIRUSES
Part 1: Biology and Epidemiology

Foodborne picornaviruses and human calicivirus have one molecule of linear, positive-sense, single-stranded RNA, covered by a capsid without a lipid envelope. These viruses have a spherical shape, a particle diameter of 27-40 nanometers and the triangulation (T=3) icosahedral symmetry of a capsid. The replication cycle of poliovirus (PV) and hepatitis A virus (HAV) occurs in the cytoplasm of the host cell. The viral proteins are synthesized by the translation of RNA after the virus particles attach and uncoat at the host cell membrane. Major translated proteins are structural protein (capsid protein) and functional proteins e.g. proteases and RNA-dependent RNA polymerase. The capsids of PV and HAV comprise multiple capsid proteins (VPs), but there is only a single structural protein in the calicivirus capsid. A distinctive characteristic of the capsid architecture is the cup-shaped depressions of caliciviruses. The RNAs of Noroviruses (NV) and feline calicivirus are organized into three major open reading frames (ORFs).

The HAV, PV and NV are transmitted enterically. These viruses are recognized as major causes of foodborne and waterborne disease in the U.S. The leading method of detection is reverse transcription polymerase chain reaction (RT-PCR) since the host cells of wild type HAV and NV are not available for an infectivity test. One may speculate that NV will become the leading cause of foodborne illness because as many as 35% of foodborne outbreaks of unknown etiology may fit the clinical and epidemiological criteria that are used to implicate NV.

Keywords : Biology, Epidemiology, Picornaviruses, and Caliciviruses

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บทคัดย่อ

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การทำลายไวรัสพิคอร์น่า (Picornaviruses) และไวรัสแคลลิซี่ (Caliciviruses)
ตอนที่ 1 ชีววิทยาและระบาดวิทยา

เชื้อไวรัสพิคอร์น่า (Picornaviruses) และไวรัสแคลลิซี่คน (Human calicivirus) มีสายพันธุกรรมชนิด RNA เส้นเดียวขดอยู่ภายในแคปซิด (capsid) และไม่มีเยื่อหุ้ม (envelope) ไวรัสเหล่านี้มีรูปร่างเป็นทรงกลมเส้นผ่าศูนย์กลาง 27-40 นาโนเมตร ไวรัสเพิ่มจำนวนตัวเองในไซโตพลาสมของเซลล์โฮสต์ หลังจากไวรัสจับกับผนังเซลล์ (attachment) จะมีการอ่านและผลิตโปรตีน (translation) จากสายพันธุกรรม RNA ที่ถูกปล่อยเข้าสู่เซลล์ (uncoating) โปรตีนที่ผลิตขึ้นจะมี 2 กลุ่มหลัก คือ โปรตีนโครงสร้างที่หุ้มสายพันธุกรรม (capsid) และโปรตีนทำงาน เช่น เอนไซม์ย่อยโปรตีนและเอนไซม์ที่สร้างสายพันธุกรรม RNA แคปซิดของไวรัสโปลิโอ (Poliovirus) และไวรัสตับอักเสบเอ (Hepatitis A virus) ประกอบด้วย โปรตีนแคปซิดหลายชนิดแต่แคปซิดของไวรัสแคลลิซี่เป็นโปรตีนแคปซิดเพียงชนิดเดียว ลักษณะที่เด่นของแคปซิดของไวรัสแคลลิซี่ คือ รอยยุบลักษณะคล้ายถ้วยที่เปลือกด้านนอกของแคปซิด สายพันธุกรรม RNA ของไวรัสโนโร และไวรัสแคลลิซี่แมว (Feline calicivirus) แบ่งได้เป็น 3 ส่วนใหญ่ๆ (open reading frames)

ไวรัสโปลิโอ ไวรัสตับอักเสบเอ และไวรัสโนโรติดต่อได้โดยผ่านทางเดินอาหาร (enterically) ไวรัสเหล่านี้เป็นกลุ่มหลักที่ก่อให้เกิดโรคโดยผ่านอาหารและน้ำ ปัจจุบันนี้ยังไม่พบเซลล์โฮสต์ที่ถูกทำลายหรือฆ่า (infectivity test) โดยไวรัสตับอักเสบเอจากธรรมชาติและไวรัสโนโรได้ ดังนั้น การตรวจสายพันธุกรรมโดยวิธีการเพิ่มจำนวนส่วนหนึ่งของพันธุกรรม (reverse transcription polymerase chain reaction) จึงเป็นวิธีที่ได้รับความนิยมในขณะนี้ มีการคาดว่าไวรัสโนโร น่าจะเป็นไวรัสที่เป็นสาเหตุหลักของการระบาดของโรคโดยผ่านอาหาร เนื่องจากการระบาดของโรคโดยผ่านอาหารที่ไม่ทราบสาเหตุสูงถึงร้อยละ 35 มีลักษณะระบาดวิทยาและอาการป่วยเข้าข่ายว่าจะเกิดจากไวรัสคล้ายนอร์วอล์ค

คำสำคัญ : ชีววิทยา ระบาดวิทยา ไวรัสพิคอร์น่า ไวรัสแคลลิซี่

1. Biology of Picornaviruses

In order to get an insight into the mechanisms of inactivation and the biology of viruses, it is important to evaluate their structural and functional changes.

Picornaviruses include many pathogens known to cause disease in humans and animals e.g. hepatitis A virus (HAV), poliovirus (PV), foot-and-mouth disease virus (FMDV), rhinovirus (causing the common cold), etc. (Racaniello, 2001). Many significant milestones in modern virology are associated with picornaviruses. FMDV was the first animal virus to be identified, by Loeffler and Frosch in 1898 (Rott and Siddell, 1998). Some decades later poliomyelitis caused widespread epidemics, which prompted the discoveries of cell culture propagation and plaque assay (measuring the infectivity of virus) (Racaniello, 2001).

The picornavirus family comprises six genera: enterovirus, hepatovirus, rhinovirus, aphthovirus, cardiovirus, and parechovirus (International Committee on Taxonomy of Viruses et al., 2000[a]). The picornavirus name was coined from *pico* meaning small (the diameter of a hydrated native picornavirus is only about 30 nanometers) and RNA, which comprises the viral genome (Racaniello, 2001). The spherical virion has one molecule of linear, positive-sense, single-stranded (ss) RNA, covered by a capsid without a lipid envelope, so the infectivity is

insensitive to organic solvents. The virion molecular weight is 8-9 x 10^6 kilodaltons. The sedimentation coefficient (S_{20W}) is 140-165S and the buoyant density in CsCl gradient is 1.33-1.45 g/cm^3. The lengths of the RNA molecule are 7.2-9.4 kb (Racaniello, 2001), containing a single long open reading frame (ORF). A small, genome-linked virion protein (VPg) and a polyA tract are covalently attached to the 5' and 3' ends of the ssRNA genome, respectively(International Committee on Taxonomy of Viruses et al., 2000[a]). The triangulation (T = 3) icosahedral symmetry of the capsid contains 60 identical copies of three or four viral proteins (VPs) (International Committee on Taxonomy of Viruses et al., 2000[a]).

One distinguishing feature of enteroviruses (Salo and Cliver, 1976) and even more of hepatoviruses (Siegl et al., 1984), is their extraordinary acid stability. At room temperature, at pH 1.0, PV type 1 vaccine strain (PV-1), human coxsackievirus types A9 (CV-A9) and B1 (CV-B1) and human echovirus type (E-9) lose almost 7 $\log_{10}$ infectivity within 2 hrs, whereas highly purified HAV remains infectious for up to 8 hrs (Scholz et al., 1989). In contrast, the rhinoviruses and aphthoviruses are acid-labile below pH 5-6 (International Committee on Taxonomy of Viruses et al., 2000[a]). This discrepancy is selectively predicated by two major factors: the sites of virus replication inside the host's body and the physical properties of the capsid. Rhinovirus and aphthovirus replicate in the host's respiratory tract, so the need for acid stability is minimal. In order to gain access to the host's intestine, enterovirus and hepatovirus need to pass through a high level of acid in the stomach, so these viruses are more acid-resistant than rhinoviruses and aphthoviruses. Otherwise, the genome organization and nucleotide sequence homogeniety of HAV and rhinovirus are very similar (see also the genome organization) (Brinton and Rueckert, 1987), so the only component that determines stability is the capsid.

1.1 Physical properties

Poliovirus (PV) is the type species of the genus enterovirus in the picornaviridae family (International Committee on Taxonomy of Viruses et al., 2000[a]). PV has a spherical shape and a particle diameter of 28-30 nanometers (nm). The total dry molecular weight is 8.25x10^3 kilodaltons. S_{20W} and buoyant density in CsCl gradients is 150-160 S and 1.34 g/cm^3, respectively. Two isoelectric points (pI) of PV type 1 are pH 7.0 (A-form) and pH 4.5 (B-form) (Mandel, 1971). Diffusion coefficient (D^o20, W 1x10^{-7}) is 1.44 cm^2/s.

HAV is the type species of the genus hepatovirus in the picornaviridae family (International Committee on Taxonomy of Viruses et al., 2000[a]). The particle size is 27-32 nm. HAV has a S_{20W} of 156-160 S and a buoyant density of 1.32-1.34 g/cm^3 in CsCl (Racaniello, 2001). HAV is stable at 60^{o}C for 60 min (Siegl et al., 1984).

1.2 Genome organization

The genome organizations of picornaviruses have a pattern of three regions: 5'nontranslated regions (NTR), ORF, and 3'NTR (International Committee on Taxonomy of Viruses et al., 2000[a]).

For PV, VPg is covalently linked to the 5'-uridylylate moiety at the 5'terminus by an O4-(5'-uridylyl)-tyrosine linkage. VPg is singly encoded in all picornaviruses but encoded in three VPg genes for FMDV. VPg is not required for RNA infectivity and is also not found on viral RNA associated with ribosomes, since VPg is cleaved off the viral RNA by a cellular enzyme (unlinking enzyme), whereby viral RNA becomes viral mRNA (Ambros and Baltimore, 1980).

At both termini, the infectious ssRNA is flanked with the NTRs which contain some secondary structures (Racaniello, 2001). These folding structures are indispensable to the viral replication cycle. At the 5'end, aside from VPg, the very long 5'NTR contains, in the 5'-terminal domain, a so-called "clover-leaf" in poliovirus, which is involved in the initiation of viral protein and RNA synthesis. Between the cloverleaf and the translation start sites, there is another secondary structure called an

internal ribosome entry site (IRES). Two IRES groups are characterized by the secondary structure: type I (found in rhinovirus and enterovirus) and type II (aphthovirus and cardiovirus). The IRES of hepatovirus is different from these two types so it might warrant a type III designation. The IRES structure makes cellular ribosomes bind directly with the internal site of the viral RNA; otherwise the ribosome scans from the 5'terminus as in routine cellular (cap-dependent) translation. The 3'NTR contains a pseudo-knot structure and may control viral RNA synthesis (Jacobson et al., 1993). After the IRES stimulated translation initiation, the polyprotein precursor is processed into P1, P2 and P3 regions containing 4, 3 and 4 proteins (4:3:4), respectively. The structural proteins, known as viral protein 4 (VP4; which is not found in hepatovirus), VP2, VP3 and VP1 are derived from the P1 region. Some functional proteins from the P2 and P3 regions are proteases, VPg and viral RNA-dependent RNA polymerase.

Like the genome of the typical picornavirus, the HAV genome is divided into three regions: 5'NTR, ORF, and 3'NTR (Racaniello, 2001). Despite the fact that the lengths of the HAV genome regions are similar to those of other enteroviruses (Ticehurst, 1986), the nucleotide sequence of HAV is not homologous with those of other picornaviruses (Brinton and Rueckert, 1987). Further, more the G+C content (38%) in HAV is about as low as that of rhinovirus (39-40%). The preference of either A or U in the third base position of codons is common in both HAV and rhinovirus (Brinton and Rueckert, 1987). The 5'NTR, which is uncapped, includes about 10% of the genome and is linked to VPg with a covalent bond. This 5'NTR of HAV seems to be the most conserved region of the genome and contains some important features; stem-and-loop structure, pyrimidine-rich tract, hypervariable tract, IRES, conserved tract, oligopyrimidine tract (also highly conserved) and translation initiation. A high percentage of C and U nucleotides is found near the 5'terminus. HAV propagation can be stimulated by some mutations in the 5'NTR (Day et al., 1992; Emerson et al., 1992; Funkhouser et al., 1994). The nucleotides 96-139 are not responsible for viral replication in cell culture (Shaffer et al., 1994) or HAV virulence (Shaffer et al., 1995). However the deletion of nucleotides 140-144 renders HAV temperature-sensitive and HAV becomes noninfectious when nucleotides 93-143 are deleted (Shaffer et al., 1994). Some cellular proteins suppress the HAV's RNA synthesis by binding at the 3'NTR (Nuesch et al., 1993). The effect of 5'and 3'sequences on picornaviral replication is limited, but it is thought to be associated with the secondary structure and virus replication (Harmon et al., 1991).

1.3 Replication cycle

Replication of PV occurs entirely in the cytoplasm of the host cell. The primary hosts of enteroviruses and hepatoviruses are humans and other mammals (Racaniello, 2001). The first step is so-called attachment: the interaction of the virus receptor-attachment site with the PV receptor (PVr) for PV and HAVcr-1 receptor for HAV. The RNA genome is then uncoated, which involves structural changes in the capsid. Once the positive-strand viral RNA enters the cytoplasm, it is translated to supply viral proteins, which are necessary for genome replication and new virus particle production. The viral proteins are synthesized from a polyprotein precursor, which is cleaved nascently. Cleavages are carried out mainly by two viral proteinases. Among translated proteins are the viral RNA-dependent RNA polymerase and accessory proteins required for genome replication and RNA synthesis. For the first step of genome replication, the released positive-stranded RNA is the template for the transcription of a negative-stranded intermediate; this step is followed by transcription of additional positive strands. These events take place on small membranous vesicles that are induced by translated accessory virus proteins. The encapsidation process is initiated when the capsid protein pool is adequate. Coat protein precursor P1 is cleaved to form an immature protomer, which then assembles into pentamer. These pentamers assemble with newly synthesized, positive-stranded RNA to form the

progeny virus particle.

Unlike PV, HAV usually does not kill but chronically infects the host cell (Binn et al., 1984). Three stages of HAV replicative events have been recognized in persistently-infected cells under a one-step growth condition (De Chastonay and Siegl, 1987). During the first week after inoculation, replicative intermediate, positive-strand RNA and progeny-virus production rates reach their peak levels. By week 2, viral antigen production is at its highest level and positive-strand RNA and progeny virus levels remain high but replicative intermediate becomes undetectable. After week 2, there is little viral metabolic activity. Several mechanisms have been proposed to explain the typically slow cycle of HAV replication as opposed to the aggressive replication cycle of PV. First, inefficient uncoating is caused by incomplete maturation cleavage of VP0 (VP4/VP2 fusion protein) from the previous cycle (Bishop and Anderson, 1993; Wheeler et al., 1986). Secondly, replication is not synchronized in certain cells (Cho and Ehrenfeld, 1991). Thirdly, translation of HAV competes poorly with that of cellular mRNAs because, unlike PV, wild type HAV does not interfere with the host-cell translation machinery (Glass et al., 1993). Fourthly, a posttranslational step e.g., a RNA-dependent cleavage of VP0 capsid protein in provirions of hepatitis A virus, may be ineffective (Bishop and Anderson, 1993). Fifthly, viral RNA may be encapsidated by HAV capsids and become unavailable as a template for translation or replication (Brinton and Rueckert, 1987). Sixthly, host-mediated, HAV, down-regulation may occur (Nuesch et al., 1993). These mechanisms may be interdependent.

1.4 Translated proteins

When PV ssRNA is free within the host-cell cytoplasm, positive-stranded genomic RNA has to be translated because it cannot be made by any cellular RNA polymerase and no viral enzymes are brought into the cell within the viral capsid (Racaniello, 2001). The positive-stranded RNA genome of PV lacks a 5'terminal cap structure; although virion RNA is linked to VPg, this protein is removed by a cellular "unlinking" enzyme on the entry of the RNA into the cell (Ambros and Baltimore, 1980). Moreover, the PV genome is efficiently translated in the cellular cytoplasm, regardless of inhibition by cellular mRNA translation. The nucleotide sequence of PV positive-stranded RNA reveals a 741-nucleotide 5'NTR region that contains seven AUG codons (Kitamura et al., 1981; McKnight and Lemon, 1998) which is the starting codon of protein translation. Such multiple protein translation-initiation codons in 5'NTRs that were subsequently found in other picornaviruses, also contain a highly ordered RNA structure (Rivera et al., 1988; Skinner et al., 1989). These multiple AUG codons and secondary structures at the 5'NTR suggest that ribosomes do not scan through the PV 5'NTRs but bind to an internal sequence of 5'NTR. The 5'NTR of PV positive-stranded RNAs is consequently thought to contain a sequence that promotes translation initiation by IRES.

PV proteins are synthesized by the translation of a single, long, open reading frame (ORF) on the viral positive-stranded RNA genome; later, the polyprotein is cleaved by virus-encoded proteinases (Racaniello, 2001). The nomenclature of translated protein is the following: VP4, VP2, VP3 and VP1 in the P1 region, 2A, 2B and 2C in the P2 region, and 3A, 3B, 3C and 3D in the P3 region. This approach not only allows the production of several proteins from a single RNA genome but also allow the non-uniform synthesis of viral proteins. During the earlier periods of the replication cycle, the viral coat proteins, coded by the RNA coding region near the 5'end, are accumulated at a higher rate than proteins coded at the other end of the RNA coding region (Paucha et al., 1974; Koch et al., 1980). The complete-length polyprotein is not found in infected cells because it is processed once the protease-coding sequences have been translated. The polyprotein precursor is processed co-translationally by intramolecular (in *cis*) reactions in what are called primary cleavages, followed by secondary intramolecular or intermolecular processing (in *trans*). PV genomes

encode two proteinases: $2A^{pro}$, and $3C^{pro}$. In cells that are infected with enteroviruses or rhinoviruses, the primary cleavage between P1 and P2 is mediated by $2A^{pro}$ but in hepatoviruses and parechoviruses the $2A^{pro}$ protein does not have any proteolytic activity and has no known function. The primary cleavage between the capsid protein precursor and the P2 region of these viruses is carried out by $3C^{pro}$ (Jia et al., 1993; Schultheiss et al., 1994; Schultheiss et al., 1995). Protein 2B is accountable for the proliferation of membranous vesicles that are the sites of viral RNA synthesis; the 2B protein also induces cell membrane permeability, which may play a part in discharging progeny viruses from infected cells (Aldabe et al., 1996; Sandoval and Carrasco, 1997; van Kuppeveld et al., 1997[a]; van Kuppeveld et al., 1997[b]). Protein 2C is involved in PV RNA synthesis and has been implicated in the process of establishing an organized sub-cellular site for PV RNA synthesis and of binding the RNA replication complex to the membrane (Teterina et al., 1997). Protein 3AB is responsible for the synthesis of poliovirus, (+) polarity, RNA, the uridylylation of VPg and anchoring the RNA replication complex to the membrane during the priming step of RNA synthesis (Paul et al., 1994; Plotch and Palant, 1995; Richards and Ehrenfeld, 1998). Protein 3D is the RNA-dependent, RNA polymerase.

1.5 Capsid proteins

The capsid of PV is composed of four structural proteins: VP1, VP2, VP3, and VP4 (Racaniello, 2001). The capsid shell is shaped by VP1 to VP3 and VP4 lies on its inner surface. VP1 to VP3 have no sequence homology, yet all three proteins have the same topology: they outline an eight-stranded anti-parallel β-barrel (β-barrel jelly roll or Swiss-roll β-barrel). This domain is a wedge-shaped structure made up of two anti-parallel β-sheets. One β-sheet forms the wall of the wedge and the second, which has a bend in the center, forms both the wall and the floor. The wedge shape enables the packing of structural units to form a dense, rigid protein shell. The packing of the β-barrel domain is reinforced by a network of protein-protein contacts on the interior of the capsid, predominantly around the 5-fold axes. This network, which is shaped by the N-terminal extensions of all VPs, is critical for the stability of the virion. The main structural differences between VP1 and VP3 lie in the loops that connect the β-barrel and the N- and C- terminal sequences that extend from the β-barrel domain. These amino acid sequences give each picornavirus its distinct morphology and antigenicity.

Among detected proteins of HAV are precursor protein VP0 (which is a VP4/VP2 fusion protein), VP1 and VP3 (Hollinger and Emerson, 2001). While the VP4 is encoded in the RNA genome of HAV, it is not found in gel electrophoresis since it is in the form of the fusion protein VP0 (VP4/VP2). The three major capsid proteins are thought to share the same β-barrel domain with other picornaviruses; however alignment of amino acid sequences is complicated because most HAV proteins, particularly the capsid proteins VP1 and VP4, are distinct from those of picornarviruses. This noteworthy structural difference imparts more stability to HAV than other picornaviruses but a seeming drawback of the non-appearance of VP4 is the inefficiency of capsid assembly (Probst et al., 1999).

1.6 Virion capsid

The surfaces of PV have a wavy topography; there is a prominent star-shaped plateau (mesa) at the five-fold axis of symmetry that encloses a deep depression (*canyon*) and another protrusion at the three-fold axis (Racaniello, 2001). It was at first anticipated and then demonstrated, that the canyon is the receptor-attachment site of PV. However not all picornaviruses have canyons. (See the sections describing attachment and antigenicity.) The network formed by the N-termini on the interior of the capsid contributes considerably to its stability. At the five-fold axis of symmetry, the N-termini of five VP3 molecules form a cylindrically parallel β-sheet. This structure is connected to the N-termini of VP4 and VP1. The myristate group attaches to the N-terminus of VP4 and mediates the interaction between these two structures (Chow et al., 1987). Interactions among pentamers

(capsid subunits) are stabilized by β-strands of the VP3 and the N-terminus of VP2 from a neighboring pentamer (Filman et al., 1989). Additionally, the stability of the PV capsid is strengthened by the interactions with the RNA genome; however little is known about the arrangement of the RNA genome inside the virion.

1.7 Attachment

The first step of the replication cycle is attachment (Racaniello, 2001). This process takes place on the plasma membrane and requires an interaction between a receptor-attachment site on the virus surface and a host cell receptor. *Adsorption* indicates that a virus reversibly and complementarily interacts with the host-cell receptor without changes in either component. *Attachment* indicates the sum of the reactions leading to a rigid and firm binding of virion and the invasion of the virion into the cell membrane. At favorable temperatures, this binding is so fast that the individual steps cannot be distinguished and so both adsorption and attachment are often used synonymously (Koch and Koch, 1985[b]).

The *receptor* of PV was identified as an Ig-like receptor (PVr) in 1989 (Mendelsohn et al., 1989). The Frp/3 cell receptor for HAV may consist of phospholipids, protein and galactose (Seganti et al., 1987); however a recent discovery shows that the African green monkey kidney cell receptor of HAV, is a mucin-like, class I, integral membrane, glycoprotein (Kaplan et al., 1996). Thus, PV and HAV have some receptor types in common, along with some rhinoviruses, coxsackieviruses and echoviruses (Racaniello, 2001). Unlike coxsackievirus A21, PV and HAV do not require a second molecule or coreceptor for entry (Shafren et al., 1997). The capsid proteins of PV and HAV are arranged or formed similarly but the surface is topographically different (See the sections on capsid protein and virion capsid.) The receptor-attachment site for PV is the canyon, which interacts with the PVr of the host cell. Some models suggest that only domain 1 of PVr penetrates the canyon. It was originally thought that the structural hindrance due to the depth and small opening-diameter of the canyon would not allow the penetration of an Ig-like molecule (Rossmann, 1989); however study of a rhinovirus-antibody complex shows that the antibody penetrates deep down into the canyon (Smith et al., 1996).

At temperatures less than 25°C, PV adsorption is a relatively loose binding reaction, and PV can detach from the receptor sites in an intact form (Koch and Koch, 1985[b]). The adsorption is relatively slow at temperatures below 20°C; but the rate of adsorption is enhanced to the normal level by the presence of NaCl (Koch and Koch, 1985[b]). At temperatures above 20°C, the attachment is more dynamic, since the membrane proteins diffuse freely in the lipid bilayer of the plasma membrane and more than one receptor may participate in the process (Lonberg-Holm et al., 1975; Koch and Koch, 1985[b]). The virus particle may become inactivated (liberation of VP4) and be released (backed off) again from the membrane or it may become bound to the membrane so tightly that it can only be detached again with non-ionic detergent (Fenwick and Wall, 1973). Once the PV is locked into isoelectric point (pI) 4.5, its hydrophobic form increases its cell-membrane affinity; it appears that this condition is also more stable against the liberation of VP4.

Both calcium and magnesium divalent cations play a crucial role in the attachment of HAV; the calcium ion may affect the conformation of capsid protein (Stapleton et al., 1991; Bishop and Anderson, 1997). An acidic pH is more favorable; 20-fold more HAV attaches to BSC-1 cells at 4°C when the pH lowered from 6.5 to 5.5 (Bishop and Anderson, 1997). Acidic pH and calcium dependency are independent (Bishop and Anderson, 1997). The process of attachment of infectious HAV is independent of temperature, in the 4°C to 37°C range (Stapleton et al., 1991; Bishop and Anderson, 1997). The RGD (arginine-glycine-aspartate) sequence is the immunodominant, neutralization, antigenic site of foot-and-mouth disease virus (Fox et al., 1989) and also contributes to cell attachment; however RGD is not involved in HAV attachment to cells (Cohen et al., 1987;

Stapleton et al., 1991).

1.8 Serotypes

Although picornaviruses are simple RNA viruses with limited genetic material and structural constraints, picornaviruses have more than 217 serotypes (Rueckert, 1996). A serotype is characterized by the ability of a "monospecific" antiserum to neutralize viral infectivity (Knipe et al., 1996). Monospecific refers to an antiserum raised in a host not previously exposed, to a certain "related" virus. Serotype and neutralization are closely related and enable us to understand the protective immunity within a genetically-related virus species. No single atomic structure seems to be serotype-determinant, so serotype variability is more correlated with the interaction between virus structure and host immunity. The identification of antigenic sites on the surface of the virion is important for studies on the mechanism of neutralization and as a foundation for understanding the molecular basis of serotyping. In this regard, monoclonal antibodies have been helpful in identifying antigenic sites, because each monoclonal antibody recognizes a specific site on the virion surface and so sequencing, escape mutants can pinpoint a change of amino acid and the topography of the virion surface (Rossmann et al., 1985).

Native polioviruses are antigenically serotype specific (designated "N" or "D") whereas "A" particles inactivated by heat, exhibit group (genus enterovirus) specificity (designated "H" or "C") There are three serotypes of PV: PV type 1 (PV-1), PV type 2 (PV-2) and PV type 3 (PV-3) (International Committee on Taxonomy of Viruses et al., 2000[a]) and the difference may reflect selective adaptation to antibody-pressure from the hosts (Koch and Koch, 1985[a]).

Only a single serotype of HAV represents the hepatoviruses (Prasad et al., 1994). HAV that is isolated from a specific location or outbreak is defined as a *strain*. However, more than a single strain of HAV can be isolated from the same location; likewise the same HAV strain can also be isolated from different locations (Robertson et al., 1992). By sequencing the HAV isolates, the source of outbreaks can be epidemiologically investigated. (Niu et al., 1992). Analysis of a 168-base region of 152 HAV strains resulted in seven genotypes (I to VII) and two of the genotypes were divided into subgenotypes A and B (Racaniello, 2001). However, more recently the International Committee on Taxonomy of Viruses has classified HAV as two different strains: Human hepatitis A virus (HHAV) and Simian hepatitis A virus (SHAV)

1.9 Antigenicity

When native or infectious PV (N antigen) is heated to 56°C in saline with a pH of 7.0, for 2-3 min, the RNA is freed from the particle and the capsid acquires a new antigenicity (H antigen). However, a study shows that after native viruses are heated to 72°C, the RNA of the thermal-inactivated PV-1 and HAV, is associated with the virion capsid and thus protected from ribonuclease (Nuanualsuwan and Cliver, 2002). The immune cross-reaction between the H and N components is small or not detectable (Roizman et al., 1959). Thus antiserum raised against H antigen does not neutralize the infectivity of the native virions nor does it bind to them. In the end, heat-inactivated PV cannot attach to cells since all of its characteristic surface antigens disappear. Loss of RNA and antigenic transformation are independent events, so the antigenic transformation without the loss of encapsidated RNA, can be achieved in a shorter times by heating to 56°C or by treatment with UV light (Katagiri et al., 1967; Roizman et al., 1959). The UV dose required for the N to H conversion is greatly in excess of that needed for the inactivation of infectivity (Katagiri et al., 1967; Mapoles et al., 1978; Wetz et al., 1983). The transformation of N and H antigenicity is permanent and concerted, i.e. particles are either antigenically N or H. This implies that once the N to H transformation begins, it is quickly and completely propagated throughout the particle. This all-or-none change of all subunits, can also be called "concerted reorientation."

HAV is distinct from other known picornaviruses in both nucleic acid and amino acid sequences (Linemeyer

et al., 1985; Najarian et al., 1985; Cohen et al., 1987; Paul et al., 1987) but as HAV strongly conserves amino acid sequences, this may lead to a significant absence of antigenic variation among different HAV isolates (Lemon and Binn, 1983). Antigen of the intact HAV seems to be conformational and usually different from those of isolated HAV proteins (Gerlich and Frosner, 1983). Antibodies to purified capsid proteins or to synthetic peptides have weak or no detectable neutralizing activity except for two peptides in VP1 (11-25) and VP3 (Emini et al., 1985; Bosch et al., 1998). The immunodominant neutralization sites of HAV strain HM175 are overlapped and closely located in VP1 (Ser102, Val171, Ala176 and Lys221), VP3 (Gln 74, Asp70) and the continuous epitope VP3 (110-121), on the surface of HAV (Stapleton and Lemon, 1987; Ping et al., 1988; Lemon et al., 1991[b]; Ping and Lemon, 1992; Bosch et al., 1998; Pinto et al., 1998). Using chimpanzee monoclonal antibodies virtually identical to human immunoglobulins, competition assay confirmed previous epitopes in VP1 and VP3 and also one non-overlapping epitope (Schofield et al., 2002). There is also a possibility of a second immunodominant neutralization site (Stapleton and Lemon, 1987; Ping and Lemon, 1992). The immunogenic site may be located within the peptide loops that connect the β-strand of the capsid proteins and project from the virion, analogous to that of other picornaviruses (Ping and Lemon, 1992). Trypsin treatment of native HAV changes the reactivity and neutralization with monoclonal antibody used to define the immunodominant site, does not affect HAV infectivity (Ginsberg, 1988); however a similar study did not report any antigenic change, but found that trypsin cleaved the VP2, which is not part of the immunodominant sites (Lemon et al., 1991[a]).

2. Biology of Calicivirus

According to the taxonomic classification, the family Caliciviridae has 4 genera; Lagovirus, "Noroviruses (NV)," "Sappoviruses (SV)," and Vesivirus (International Committee on Taxonomy of Viruses et al., 2000[b]). Both NV and SV are human caliciviruses. The classical human calicivirus, that is represented by Sappovirus and the small round structured viruses (SRSVs) that are represented by Norovirus, are not only different in structure (Caul and Appleton, 1982) but also by sequence comparison ---- classical human calicivirus shares higher sequence homology with known animal calicivirus then with SRSVs (Matson et al., 1995). Feline calicivirus (FCV) is a member of the genus Vesivirus (International Committee on Taxonomy of Viruses et al., 2000[b]).

2.1 General properties

Virions of this family are nonenveloped and have icosahedral symmetry. Their diameters, by negative-stain electron microscopy, are 27-40 nm. The main structural proteins (capsid protein) arrange on a T = 3 icosahedral symmetry from 90 dimers to form the capsid. A distinctive characteristic of the capsid architecture is the 32 cup-shaped depressions, one at each of the icosahedral fivefold and threefold axes. The molecular weight of a virion is about $15x10^6$ kilodaltons. Virion buoyant density is 1.33-1.41 g/cm^3 in CsCl and virion S20W is 170-187S. The genomic RNAs consist of a linear, positive sense, ssRNA molecule of 7.4-8.3 kb. The genomic RNAs of Norwalk and Southampton viruses are organized into three major ORFs; this organization is similar to that of FCV (Carter et al., 1992). The 5'region encodes nonstructural protein, while the structural proteins are encoded in the 3'region. The genomic RNA acts as template for polyprotein translation and premature polyprotein is nascently cleaved by a gene-encoded protease to form the mature nonstructural proteins. VPg is covalently attached to the 5'terminus of the RNA and the 3'terminus has a polyadenylated (poly A) tail. In the host cell cytoplasm, subgenomic RNA is 2.2-2.4 kb and predominantly used to translate capsid protein (CP) which is approximately $5.8\text{-}6.0x10^4$. One minor protein is found only in Lagovirus. Nonstructural proteins have homology with those of the family Picornaviridae; 2C helicase, 3C protease and 3D RNA-dependent RNA polymerase domains that have

been previously identified in FCV (Neill, 1990).

2.2 Distinctive features of feline calicivirus

The genomic RNA is structured into three major ORFs. ORF1 encodes the nonstructural polyprotein. ORF2 encodes the major structural CP that is translated as a larger precursor protein and then cleaved into mature CP. The ORF1 and ORF2 of FCV are separated by only two nucleotides (GC) while the ORF1 and ORF2 of NV overlap with approximately 10-17 nucleotides. The ORF3 encodes a small protein with unknown function and overlaps by one nucleotide with ORF2 in a -1 frameshift of protein translation. The protein translated from ORF3 has been identified in cells infected with FCV (International Committee on Taxonomy of Viruses et al., 2000[b]).

Human caliciviruses are not successfully propagated in cell culture e.g., human embryonic intestinal organ culture (Kapikian, 1994; Schmidt and Emmons, 1995). FCV and most other members of the genus Vesivirus can be propagated in cell culture. FCV grows most efficiently in cells of feline origin and upper respiratory tract or kidney cells (Tenorio et al., 1991). FCV is used in inactivation studies as a surrogate for NV (Doultree et al., 1999).

2.3 Capsid and antigenicity

The protein capsid of the Noroviruses contains the antigenic sites. When the genes that encode the Norovirus capsid protein (ORF2) and the terminal ORF3 protein, are artificially expressed in cells, the capsid protein readily assembles into recombinant, virus-like, particles (rVLPs) without encapsidating genomic RNA(Jiang et al., 1992). The rVLPs of the Norwalk virus are useful for the study of the capsid's structures (Prasad et al., 1994; Prasad et al., 1999). The calicivirus capsid has 90 dimers of the major structural proteins that form the structure of the capsid shell, from which 90 archlike capsomeres protrude to the virion surface. These arches are organized to form large hollows at the icosahedral 5- and 3-fold positions; these hollows are cuplike structures on the surface of caliciviruses (calici from calyx meaning cup) (Green et al., 2001). The capsid has two domains: the shell (S) and the protruding arm (P). The S domain forms the internal layer of the capsid, covering the genomic RNA and the P domain, forms the archlike capsomeres that protrude from the shell. The amino terminals (N), which are located within the S domain, consist of residues 10-49 and point in toward the virion core. The S domain that forms a β-barrel, consists of amino acid residues 50-225. The entire S domain (1-225) constitutes the N-terminal region of the capsid protein and is also comparatively conserved among NLVs in sequence comparisons. The P domain is divided into P1 and P2 subdomains. Two P1 subdomains sit on top of the S domain and erect the P2 subdomain protruding from the top of the arch.

The variable region (P2 subdomain) of the capsid is consistent with the configuration of a major antigenic site and receptor binding (Hardy et al., 1996; White et al., 1996; Prasad et al., 1999). The variable region 2 of NLVs is analogous to the E variable region of FCV capsid proteins, which is also thought to be involved in the neutralizing antigenic site (Tohya et al., 1997; Neill et al., 2000).

2.4 Comparison of Picornavirus and Calicivirus

Both picornavirus and calicivirus have one molecule of linear, positive-sense, single-stranded RNA, covered by a capsid without a lipid envelope. The diameters are essentially the same. Although the genome organizations do not show the same pattern (or order), the calicivirus nonstructural proteins show homology with those of the family Picornaviridae, as mentioned earlier (Neill, 1990). The capsids of PV and HAV comprise multiple capsid proteins (VPs), but there is only a single structural protein in the calicivirus capsid; however subunit proteins of both families arrange in the same T = 3 icosahedral symmetry lattice. The hepatitis A and NV are transmitted enterically via food and water (Block and Schwartzbrod, 1988; Cliver, 1990); before infecting the host's body, these viruses must be protected in their passage through a harsh environment. Thus, through evolution, foodborne or waterborne disease viruses became resistant to the

stresses in both the host body and the environment. In view of the fact that the phylo-genetic relationship of capsid gene and nucleic acid sequences of these two families are distant (International Committee on Taxonomy of Viruses et al., 2000[b]), the protective property of both HAV and NLVs perhaps lies in the capsid stability of the virion particles whose symmetry and subunit domain are similar.

3. Epidemiology

The transmission of all known foodborne and waterborne disease viruses is via the fecal-oral route (enterically), so these viruses are shed in feces and the transmission cycle is completed by the ingestion of virus-contaminated feces directly or indirectly (Block and Schwartzbrod, 1988; Cliver, 1997). The most important source of viral infection perhaps is the transmission *directly* from person to person or direct ingestion of virus perorally. The clinical symptoms of NV infection include nausea and vomiting (American Public Health Association et al., 1995; Chin and American Public Health Association, 2000) so the virus may also be shed with vomitus (Cliver, 1997). Alternatively, these viruses can be transmitted *indirectly* via contaminated objects e.g. vectors, fomites (any objects, such as clothes or bedding that are capable of absorbing and transmitting infecting organisms), foods and water (Cliver, 1997).

Among viral foodborne or waterborne diseases, HAV infection is the notifiable one in the USA and the reported total foodborne and waterborne cases are comparable (CDC, 1999[a]). The reported cases of HAV infection are collected and reported annually by the Center for Disease Control and Prevention (CDC). Statistically, reported cases of HAV infections have fluctuated during 1988-1998, except in 1989-1990 when the incidence rates climbed to 12-14 cases per 100,000 U.S. population. In terms of the number of reported food-disease outbreaks, during 1993-1997 (Olsen et al., 2000) HAV fell to the sixth leading cause, from the fourth leading cause during 1988-1992 (Bean et al., 1996). Following the same trend for the past few years, reported cases of HAV declined to the lowest ever recorded in 1999, with 6.25 cases per 100,000 U.S. population (CDC, 1999[a]). By the end of 1999, routine childhood HAV vaccination was recommended by the Advisory Committee on Immunization Practices (ACIP) (CDC, 1999[b]) in the 11 states where the average HAV incidence rate during 1987-1997 was above 20 cases. Since the incidence rate of HAV infection varies year-by-year or even state-by-state, judgment as to whether this low incidence rate is attributable to the vaccination or the inherent fluctuation of the HAV infection rate is not conclusive (CDC, 1999[a]).

During 1988-1997, NV caused only 11 food borne-disease outbreaks (ca. 1% of the total number of reported foodborne-disease outbreaks) and were the ninth leading cause of outbreak-associated illnesses in that decade (Bean et al., 1996; Olsen et al., 2000). However it was estimated that each year NV cause more than 60% of total foodborne illnesses (Mead et al., 1999). The reported numbers are much lower than estimated because of the lack of testing serum for NV antibodies (which comes only from human volunteers), a laboratory host (or cell line) to cultivate NV and the facilities to perform the diagnostic tests. Thanks to the development of NLV detection by immunologic assays (Greenberg et al., 1978; Treanor et al., 1988), reports of foodborne outbreaks caused by NV increased considerably, to 136 during 1996-2000 (CDC, 2001). However as many as 35% of foodborne outbreaks of unknown etiology may fit the clinical and epidemiological criteria that are used to implicate NV in outbreaks of acute gastroenteritis without laboratory diagnosis (Bean et al., 1996). These criteria are the absence of bacterial and parasitic pathogens in stool samples, more than 50% of cases involving vomiting, mean (or median) sickness duration of 12-60 hours and if determined, mean (or median) incubation periods of 24-48 hours (Kaplan et al., 1982[a]). One may speculate that NV will appear in the surveillance statistics for foodborne disease outbreaks in U.S. during 1999-2003, higher than the ninth leading

cause.

Detection of viruses in clinical specimens, food and water samples is usually attempted when epidemiological investigations are involved (Cliver, 1997; Jaykus, 1997). Some traditional detection methods are burdensome, costly and often unavailable or unattempted for many important food and waterborne, enteric viruses. However with recent development in detection methods, regulatory testing mandates and epidemiological surveillance for food and waterborne disease, we may see a significant increase in the ability to detect and control these causative agents.

For clinical specimens, the epidemiological data-incubation period, sickness duration, classical, clinical symptoms caused by virus infection and the lack of bacterial or parasitic pathogens in stool samples - are useful tools for investigation (Kaplan et al., 1982[a,b]). Confirmation by laboratory methods entails further testing for the virus antigen, virus nucleic acid in stool specimens or an increase in specific antibody (IgM or IgG) to the viruses. The laboratory methods used are immune electron microscopy, radioimmunoassay, enzyme immunoassay, nucleic acid hybridization assays and RT-PCR (CDC, 2001). However, the problems are the low level of sensitivity (above 4-5 $\log_{10}$ particles/ml) and the lack of (cell culture) hosts for cultivating NV, which leads to a deficit of supplies of both viral antigen and antibody (Green et al., 2001).

Environmental, food and water samples present different problems (Jaykus, 1997). Unlike bacteria, transmissible viruses are inert or not replicative outside their specific host cells, so the enrichments routinely used in bacteriology are not applicable. Large-volume samples are usually concentrated to the optimal volume so that detection methods do not suffer from a lack of sensitivity. In reality, the concentration step is not practical and efficient enough. If detection by cell culture infectivity is applicable, this method is slow and expensive because it requires trained personnel, special facilities and long incubation periods if contaminant levels are low. These difficulties make routine virus detection difficult, however the importance of outbreak investigation outweighs the high cost of virus detection. In the unique situation of HAV, where the average incubation period is about 4 weeks, leftover food is seldom available for testing (Cliver, 1997).

Recently improved detection strategies substitute for inefficient traditional detections by infectivity and may justify a routine testing in the future (Jaykus, 1997). Two concentration schemes have been reported: Extraction-concentration methods and absorption-elution-concentration methods (Jothikumar et al., 1998). The key advances sought are higher virus yield and the absence of cytotoxic substances in the samples (Jothikumar et al., 1998). Conventionally, detection of virus after its extraction from food or water (e.g. 1-200 infectious units per gram of shellfish) (Cole et al., 1986) is dependent upon virus infectivity in cell culture and is not sensitive enough. Furthermore, some cell culture-toxic substances are inherently present in the extracted samples (Sobsey et al., 1978). However epidemiological data demonstrate that leading foodborne or waterborne viruses are not cultivatable (NV) or grow poorly (wild-type HAV) in cell cultures (Cliver, 1997; Jaykus et al., 1994). Thus, virus infectivity is no longer a realistic detection method. Enzyme-linked immunosorbent assay (ELISA) and nucleic acid probe methods slightly improve sensitivity (Jaykus et al., 1994). Reverse transcription (RT) is the step required to make complementary DNA (cDNA) from the RNA of HAV or NV, followed by specific DNA amplification by a polymerase chain reaction (PCR). The sensitivity and specificity of RT-PCR is high and it is becoming a leading molecular detection method for HAV and particularly NLVs (Jaykus et al., 1995; Cliver, 1997; Jaykus, 1997), since genome sequences of Norwalk virus and NLVs has become available (Lambden et al., 1993; Xi et al., 1990). Modifications are applied to RT-PCR to ease some problems: High sample volume, PCR inhibitor inherently present in food samples and genetic diversity among NLVs (Wang et al., 1994; Ando et al., 1995). Viral RNA from a

food sample is isolated and purified before RT-PCR (Atmar et al., 1993; Goswami et al., 1993; Gouvea et al., 1994; Lees et al., 1994; Atmar et al., 1995; Lees et al., 1995). Homologous antibody is used to capture the corresponding viruses before rigorously washing away PCR inhibitors using the immunomagnetic capture RT-PCR method (IC-PCR) or antigen capture, RT-PCR method (AC-PCR) (Desenclos et al., 1991; Deng et al., 1994; Jothikumar et al., 1998). The concentration of virus particles separated from food results in small sample volumes without PCR inhibitors and then heat degradation of the virions makes RNA available for RT-PCR (Chung et al., 1996; Jaykus et al., 1996). Even if molecular detection by RT-PCR is positive, the interpretation is that the food is contaminated with specific viruses, which may or may not be the cause of sickness in consumers. Since both infectious (potentially pathogenic) and inactivated (no longer infectious) virus may yield positive RT-PCR results; it may or may not represent a threat to health. It is more desirable to be able to demonstrate and distinguish infectious virus as positive RT-PCR and inactivated virus as negative RT-PCR. Fortunately, a pretreatment of viruses with a combination of proteinase K and ribonuclease, infectious and inactivated viruses yield positive and negative RT-PCR results, respectively (Nuanualsuwan and Cliver, 2002).

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