ANIMAL ENTERIC VIRUSES: GENE EXPRESSION, EPIDEMIOLOGY AND THEIR ROLE IN SHELLFISH AND ENVIRONMENTAL CONTAMINATION

DISSERTATION

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By

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ABSTRACT

Acute gastroenteritis (AGE) is one of the most common diseases worldwide. Seven to 41 percent of these are food-borne disease. Each year, foodborne diseases cause 76 million illnesses, more than 300,000 hospitalizations and 5,000 deaths in the U.S, indicating that 1 in 4 Americans develop disease and at least 1 in 1000 is hospitalized. Only 20-30% of these illnesses are attributed to known pathogens, and viral agents are estimated to account for more than 2/3 of these. Among viruses, noroviruses (NoVs) are estimated to cause 82% of the foodborne illnesses. HuNoVs have been detected in fruits, vegetables, ready-to-eat foods and seafood worldwide. Ten to 70% of outbreaks are associated with seafood and contamination occurs at pre- and post-harvest. However, no data has been collected to determine viral contamination in commercial shellfish harvesting areas throughout the U.S. coasts. In addition, although the identification of related animal enteric caliciviruses and recombinants have raised concerns for human infection or co-infection with different strains, the presence of animal strains in shellfish with potential risk for humans has not been studied. We determined the occurrence of human and animal enteric caliciviruses (HECV and AECV, respectively) in U.S. market oysters. Oysters were collected from 45 different bays along the U.S. coasts during summer and winter of 2002-2003. Twenty-percent of the samples were positive for HuNoVs GII, phylogenetically
similar to NoV GII US 95/96 subset (GII.4). Animal enteric caliciviruses were detected in 10 samples (22%). Seven of these were positive for porcine NoV (PoNoV) GII and one sample was positive for PoSaV GIII. Bovine NoVs (BoNoVs) were detected in two samples and confirmed by sequencing as NoV GIII. Different seasonal and state distributions were detected and HEVC and AECV strains were simultaneously detected in oysters from the same bay during the same season. Our results confirm the presence of HuNoV GII.4 in oysters purchased from markets supplied by licensed shippers that harvest them from bays on the East, West and Gulf coasts approved for human consumption. The simultaneous detection of HEVC and AECV raises concerns for possible human infection or co-infection with HEVC and AECV strains, favoring the opportunity for recombination and emergence of new strains relevant for the control of the disease.

The major source of contamination of water and food is the discharge of treated or partially treated waste into open waters. Similarly for agriculture, the causative agents of many infectious diseases are excreted by the fecal route from animals with acute and chronic infections, but also sometimes from clinically healthy animals. Several candidate environmentally superior technologies (cESTs) have been developed for the treatment of animal wastes to reduce their impact on the environment. We determined the presence of swine enteric viruses [PoNoV, PoSaV, rotaviruses (RV)-group A, B and C] in fresh feces or manure and evaluated the effects of different cESTs on their detection and viability. Porcine SaVs were detected in 97% of the samples by RTPCR, ELISA or both techniques,
whereas for PoNoVs 20% of the samples were positive by RTPCR. The RV-A and RV-C were detected in 67% and 44% of the samples by seminested-RTPCR and/or ELISA. After treatment PoSaV RNA was only detected in the Conventional Waste Management (CWM), and not from the cESTs. Rotavirus-A and -C RNA was detected in 4/5 and 3/4 cESTs after treatment, but infectious particles were not detected by cell culture immunofluorescence, nor were clinical signs or seroconversion detected in Gn pigs inoculated with post treatment samples. Thus only RV-A/C RNA, but no viral infectivity was detected after treatment. This is the first study to evaluate the impact of different waste treatment technologies on virus detection and viability in swine manure. The fact that RNA virus (PoSaV and PoNoV) was not detected after treatment and/or Gn pigs were not infected by post-treatment samples suggests that infectious virus would not be present in the field after treatment. However their utility should be evaluated together with their ability to reduce the impact of other factors (organic and inorganic residues) on the environment.

In general, efforts to prevent contamination and to develop effective treatments to inactivate HuNoV, as well as many molecular and immunological studies, have been hampered by the lack of a cell culture system to assay infectivity of enteric NoVs. Only PoSaV/Cowden strain and a murine NoV can be propagated in cell culture. The PoSaV/Cowden strain was adapted to serial propagation in an LLC-PK cell line by adding on intestinal content preparation (IC), derived from uninfected Gn pigs to cell culture. Bile acids were identified as active factors in IC essential for growth of PoSaV/Cowden in cell
culture by activating the Protein Kinase A (PKA) signaling pathway and down-regulating Interferon (IFN) mediated STAT1 activation. However in this case, the fact that the IC showed higher levels of activity than individual BA suggests that other components of the IC might inhibit STAT1 activation by other mechanisms or other undefined mechanisms mediated by IC or BA contribute to PoSaV/Cowden replication that will be applicable to other SaVs and NoVs. We measured gene expression profiles of LLC-PK cells in response to PoSaV/Cowden infection in the presence of IC at early and late time points post-infection. Based on the criteria of a ≥ 1.5-fold change, at 4h PI, 29 genes were differentially up-regulated and, at 8h PI, 83 genes were up-regulated and 7 were down-regulated in virus-infected cells compared to mock-infected cells. Genes clustered mainly into classes involved in cell cycle, cell proliferation and apoptosis, protein metabolism, immune and stress response and signal transduction. The late up-regulation of immune response genes and the absence of other intermediates in IFN responses suggest a block or delay of the IFN response. The late up-regulation of genes involved in apoptosis suggests that PoSaV/Cowden strain may trigger apoptosis for cell release or spread. This represents the first global study of cellular gene expression altered by calicivirus infection in cell culture, and shows an overview of the general changes in gene expression. Our findings may serve as a foundation for further analysis at the functional level to understand the mechanism of SaV infection with potential extension to NoVs.
Dedicated to my Father who always encouraged me to follow ahead,
to my Mother who always let me fly and to my Sister and Brother
who always were there for me

Dedicada a mi Padre, que siempre me animó a seguir,
a mi Madre que siempre me dejó volar y a mis Hermanos
quienes siempre estuvieron ahí por mi.
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LIST OF ABBREVIATIONS

3C-like protease (3CL\textsuperscript{pro})
Acute gastroenteritis (AGE)
Adenosine triphosphate (ATP)
Aminoacid (aa)
Animal enteric caliciviruses (AECV)
Becovirus, (Becov)
Bovine noroviruses (BoNoVs)
candidate Environmentally Superior Technology (cEST)
Canine calicivirus (CaCV)
Capsid protein (VP1),
Cholera toxin (CT)
Conventional Waste Management (CWM)
Cyclic AMP (cAMP)
Electrochemiluminescent (ECL)
Electronmicroscopy (EM)
Enteric caliciviruses (ECV)
Enzyme linked immunoabsorbent assay (ELISA)
European brown hare syndrome virus (EBHV)
Feline calicivirus (FCV)
Genogroups (Gx)
Genotypes (Gx.y)
Hepatitis E virus (HEV)
Histo-blood group antigens (HBGAs)
Horseradish peroxidase (HRP)
Human embryonic kidney (HEK)
Human enteric caliciviruses (HECV)
Human noroviruses (HuNoVs)
Immune electron microscopy (IEM)
interference (RNAi)
Interferon (IFN)
Interferon (IFN)
Internal control (InC)
Internal ribosome entry site (IRES)
Intestinal content (IC)
Intravenously (IV)
Isoelectric point (pI)
Junction adhesion molecule 1 (JAM-1)
Kilo Daltons (kDa)
Lewis a (Le^a)
micrograms (µg)
Mink enteric calicivirus (MCV)
Minor structural protein (VP2)
Monoclonal antibody (mAb)
Murine Norovirus (MNV)
National Center for Communicable Disease (CDC)
Nebraska strain (NB strain)
Newbury agent-1 (NA-1)
Norovirus (NoV)
nucleic acid sequence-base amplification (NASBA)
Nucleoside triphosphate (NTP)
Nucleotide (nt)
Orally (PO)
phosphorodiamidate morpholino oligomers (PMO)
Poliovirus (PV)
Polyethylene glycol (PEG)
Polymerase chain reaction (PCR)
Polypirimidine tract-binding protein (PBT)
Porcine Noroviruses (PoNoVs)
Primary porcine kidney (PPK)
Protein Kinase A (PKA)
Protein Kinase A (PKA)
Rabbit hemorrhagic disease virus (RHDV)
Real time RTPCR (rt-RTPCR)
Red blood cells (RBCs)
reverse line blot hybridization (RLB)
reverse transcription Loop-mediated Isothermal amplification (RT-LAMP)
Reverse transcription-polymerase chain reaction (RTPCR)
Ribonucleic Acid (RNA)
RNA-induced silencing complex (RISC)
Rotavirus (RV) group A, B and C
Rotavirus group A (RV-A)
Rotavirus group B (RV-B)
Rotavirus group C (RV-C)
Sapovirus (SaV)
short interfering RNA (siRNA)
Signal transducer and activator of transcription 1 (STAT-1)
Single-stranded, positive sense RNA (ssRNA+)
Small Round Structure Viruses (SRSV)
Snow Mountain virus (SMV)
Solid-phase immune electron microscopy (SPIEM)
Southern blot (SB)
The International Committee on Taxonomy of Viruses (ICTV)
The Ohio Department of Health (ODH)
Tissue culture-adapted PoSaV/Cowden (TC-PoSaV/Cowden)
transcriptional enhancement of RTPCR (TE/RTPCR)
Translation initiation factors (eIFs)
Untranslated region (UTR)
Vesicular exanthema of swine virus (VESV)
Virus-like particles (VLPs)
Wild-type PoSaV/Cowden (WT-PoSaV/Cowden)
CHAPTER 1

LITERATURE REVIEW ON ENTERIC CALICIVIRUSES, AND ROTAVIRUSES

1.1. ENTERIC CALICIVIRUSES

1.1.1. INTRODUCTION

“This must be a virus”. Probably these were Dr Zahorsky’s thought when describing epidemic nonbacterial gastroenteritis for the first time in 1929 (719). Named as “winter vomiting disease” this entity was characterized for sudden onset of nausea and vomiting (sometimes a prominent diarrhea) and mild fever; occurring between September and March, involving students and institutional personnel; common source, secondary spread and failure to detect a bacteria as the etiological agent. Between 1936 and 1969 at least 10 other outbreaks of “winter vomiting disease” were reported with the common characteristic: no known bacteria were detectable (66, 225, 262, 265, 298, 397, 411, 453, 525, 540, 680). In 1968, two outbreaks of winter vomiting disease were studied by the Centers for Disease Control and Prevention (CDC) and The Ohio Department of Health
The first one affected 50% of students and personnel in an elementary school in Norwalk, Ohio. Most cases occurred in the 24h between October 30 and 31 and symptoms (nausea, vomiting and abdominal cramps) persisted 12-24 h. The secondary attack rate among family of primary cases was 32%, between November 1 and 3, and a follow-up survey 1 month latter showed a significant increase in the attack rate for gastroenteritis among residents of Norwalk. However bacteriological and parasitological examination of stool swabs, food, milk and water were negative. The second outbreak was also in Ohio (City of Columbus) and also affected students and personnel at an elementary school. Overall the attack rate was 25% (with the highest rate of 88%) and secondary attack rate of 30% (5). A viral cause was suspected, but could not be proved.

Then in 1972, Kapikian et al (336) used immune electron microscopy (IEM) to identify viral particles in the stools of volunteers who were infected with a passage of the Norwalk virus (NV) inoculum. Virus particles were originally classified as Small Round Structured Viruses (SRSV) because of their appearance. Almost simultaneously a morphologically distinct SRSV called “classic human calicivirus” was described by Madeley and Cosgrove (426) in England and by Chiba et al (113) in Sapporo, Japan. Almost 20 years latter and after the genome was cloned (316), NV virus was classified as member of the Caliciviridae family in a different genus than the Sapporo strain.

1.1.2. INFECTIOUS AGENT

1.1.2.1. Agent

Caliciviruses are small, non-enveloped, single-stranded, positive-sense RNA viruses. The icosahedral viral capsid (27-40 nm in diameter) is composed of a single
major (VP1) capsid protein and it contains genomic RNA of 7-8 kb in length, excluding the poly A tract (231, 441). The characteristic “Star of David” morphology (32 cup-shaped surface depressions or chalices) by electron microscopy (EM) is the reason for the family name. However this is now a generalization, because many family members, particularly those that cause gastroenteritis in humans, have an indistinct “feathery” outer edge although there is a suggestion of indentation in the surface (Figure 1.1) (116).

1.1.2.2. **Taxonomy**

Originally, five potential subfamilies within the *Caliciviridae* family were suggested: Norwalk-like small round-structured viruses (SRSV), Sapporo-like, Hepatitis E virus (HEV)-like, rabbit- and vesicular exanthema of swine virus (VESV)-like, each with a distinct genome organization (42). However further studies by the same authors in 2000, reclassified the members of this family into four genera: *Norwalk-like virus*, *Sapporo-like virus*, strains related to rabbit hemorrhagic disease virus and strains related to vesicular exanthema of swine virus, excluding HEV from the caliciviruses (43). This classification was approved by The International Committee on Taxonomy of Viruses (ICTV) in 2000 (230). After renaming the *Norwalk-like* and *Sapporo-like* genera (441), the family *Caliciviridae* was divided into four genera based on sequence identities and genome organization: *Norovirus*, *Sapovirus*, *Vesivirus* and *Lagovirus*. Viruses in the *Vesivirus* genus cause vesicular lesions similar to foot-and-mouth disease in swine (VESV), abortion and lesions in marine mammals (San Miguel sea lion virus, SMSV) and severe respiratory illness in cats (Feline calicivirus, FCV). Rabbit hemorrhagic disease virus (RHDV) and European brown hare syndrome virus (EBHV) belong to the
Lagovirus genus and cause fatal hemorrhagic liver disease in their respective hosts (116). However none of them naturally infect or cause diarrhea in humans, whereas viruses in the Norovirus (NoV) and Sapovirus (SaV) genera cause diarrhea in humans and animals and are referred to as human or animal enteric caliciviruses (HECVs or AECVs, respectively) (231). Recently a fifth genus (Becovirus or Nabovirus genus) within the Caliciviridae family was proposed (495, 593). Members of this genus were originally detected in feces from diarrheic calves by Woode et al in the U.K in 1978 (701) and by Smiley et al in the U.S. in 2002 (593). Smiley et al first sequenced the entire genome of the NB strain in 2002 and proposed it to be a new calicivirus genus based on aminoacid (aa) identities of 14.1% and 22.6% over the non structural proteins with other calicivirus, whereas the overall sequence identity of the complete capsid with other calicivirus was between 14.6% and 26.7% (593). Only more recently (2006) the European strain, Newbury agent-1 (NA-1) was sequenced, also demonstrating less than 39% aa (47% nucleotide, nt) identity with other calicivirus genera in the complete 2C-like helicase, 3C-protease, 3D-polymerase and capsid regions, but 89-98% aa (78-92% nt) identity to Nebraska (NB) strain detected by Smiley et al in the U.S. (495).

The classification of enteric caliciviruses (ECV) (human and animal strains) has evolved with the discovery of more diverse strains. With the development of new and more sensitive techniques to detect ECV and the replication strategies used by the virus, the number of strains has grown significantly recently. Simultaneously a more detailed classification was needed. Today ECV are classified into genus, subdivided into genogroups (Gx) and further subdivided into genotypes (Gx-y) or clusters. Nucleotide sequence differences among strains within a genotype (0-15%), between genotypes (15-
45%), between genogroups (46-62%) and between genus (84-86%) have been defined based on phylogenetic analysis of partial RNA-dependent-RNA-polymerase (RdRp) and complete capsid of NoVs and SaVs (including human and animal strains) (15, 491, 571, 594, 675, 676, 720). The most significant efforts contributing to the classification of ECV are summarized in Table 1.1. The proposed nomenclature for NoVs and SaVs is: species infected/virus genus/virus name/strain designation/year of isolation/country of isolation (21). However the prototype strains of each genotype are referred to by their common name, which corresponds to the place of isolation.

Members of the Norovirus genus are genetically and antigenically diverse (15, 320, 342). Antigenic classification lacks accuracy and reproducibility, making genomic classification based on sequencing the means to characterize NoVs. Since the cloning of NV in 1990 (316), several attempts have been made to classify NoVs based on partial or complete sequence of Open reading frame 1 (ORF-1) or ORF-2. Most investigators have focused on the conserved sequence of the RdRp (encoded by the ORF-1) to design primers to detect several strains (317, 329, 381). Others have mainly focused on the partial or complete capsid sequence (230, 665) or on both RdRp and capsid sequence (172).

A complete analysis based on capsid sequences (164 strains) was performed by Zheng et al 2006 (720). The NoVs are divided into five genogroups (GI, GII, GIII, GIV and GV) and each genogroup is further subdivided into genotypes (8, 17, 2, 1 and 1, respectively) based on phylogenetic tree topology and distance analysis of the complete capsid protein (Figure 1.2) (720). The pairwise distance difference based on the complete capsid aa sequence analyzed by the uncorrected method was set as 15-45%, meaning that strains with shorter differences will be grouped into the same cluster and
strains with higher differences will belong to another existing or new genogroup. Human noroviruses (HuNoVs) belong to GI, GII and GIV, whereas GIII includes only bovine noroviruses (BoNoVs) (132, 133, 297, 409, 494, 594). Porcine Noroviruses (PoNoVs) belong to GII (621, 653) and a Murine Norovirus (MNV) that produces a systemic infection belongs to GV (339). The shortest distance among HuNoV genogroups was observed between GII and GIV (41.88-44.91%), whereas the greatest distance was observed between MNV (GV) and GI-III.

Wang et al 2005 (673) reported 2 new genotypes in GII after analyzing the complete capsid of 5 PoNoV strains detected in the U.S. These new strains belong to GII-11 (Po/NoV/MI-QW48/02/US), GII-18 (Po/NoV/OH-QW101/03/US and Po/NoV/OH-QW125/03/US) and GII-19 (Po/NoV/OH-QW170/03/US and Po/NoV/OH-QW218/03/US).

The concept of “subgenogroups” (known today as genotypes) for SaV was introduced by Jiang et al in 1997 based on antigenic differences between 5 strain members of 2 different genogroups (314). Using the same criteria and methodology, the first genetic classification of members of the Sapovirus genus was provided in 2001. The genus was divided into three genogroups (GI-III) with each subdivided into three, one and one genotype, respectively (571). Four further studies of human and animal strains increased the complexity of this classification (176, 490, 663, 676). Okada and colleagues in 2006 (491) performed a new study that included 25 human and animal SaV strains. Their results suggested that the Sapovirus genus should be divided in five genogroups (GI-V) with each subdivided in six, four, one, one, and one genotypes, respectively, based on distance and phylogenetic analysis of the capsid sequence. Human sapoviruses
(HuSaVs) belong to GI, GII, GIV and GV whereas the porcine sapovirus (PoSaV) belongs to GIII.

Among animal strains, SaVs include swine and mink strains. Mink enteric calicivirus (MCV) causes gastroenteritis and it was first detected by Guo et al in 2001 (247). The first PoSaV strain, Po/SaV/Cowden/80/US, was reported by Saif and colleagues (1980) based on its calicivirus morphology by EM in diarrheic stools of pigs in the U.S. (559) and further assigned to SaV GIII-1 (246, 571). Thereafter, other PoSaV strains were detected in the U.S. (677), Korea (350), Japan (710) and Venezuela (433). Although a complete analysis that includes all of these have not been performed, separate studies showed that strains from Korea share higher aa and nt identity (partial capsid sequence) with Po/SaV/Cowden/80/US (GIII-1), whereas Japanese strains are suggested as members of a new genogroup. Wang et al (2005) detected 9 new strains in the U.S.: six of them were assigned to GIII-1 (Cowden-like) and a potentially new genogroup was suggested for Po/SaV/OH-JJ681/00/US strain detected in postweaning pigs with diarrhea. Martinez et al (2006) suggested that 6/8 Venezuelan strains belong to GIII-1 (Cowden-like) and 2/8 defined a potentially new genotype within genogroup III. A comprehensive analysis that includes all the PoSaV strains is needed to accurately reflect their diversity.

A distinct feature between NoVs and SaVs taxonomy is the fact that the classification of strains based on analysis of the partial genome segments (RdRp or capsid) results in a different scheme for NoV (720), whereas the classification of SaVs is maintained throughout the entire genome, suggesting that SaVs are evolutionarily more stable than NoVs (176, 663). Surprisingly, despite the great diversity and co-circulation of NoV strains, usually one strain has emerged as predominate or the most common
cause of most of the outbreaks at different times (54, 365, 419, 478, 686), whereas no predominant strain has been detected among co-circulating SaV strains (571, 663).

### 1.1.2.3. **Virus evolution**

Caliciviruses infect a wide range of mammalian hosts. Co-phylogenetic analysis of these viruses (HuNoV, RHDV, SMSV, EBHV, FCV, VESV, BoNoV, and PoSaV) with their hosts (rabbit, brown hair, cow, pig, cat, sea lion and human) indicated only two host switches. An early switch between *Carnivora* (represented by cat and sea lion) and *Lagomorpha* (represented by rabbit and hare), and a later host switch from sea lion (SMSV) to pig lineages (VESV) (168). The same study also indicated that the other members of the *Caliciviridae* family showed co-divergence with their host. Recombination and selection in the evolution of RNA virus is characterized by high frequency for recombination, evidence for positive selection and differential codon use in the capsid region (589). Several theories for the evolution and maintenance of virulence have been developed (396). A classical trade-off model of virulence evolution has been proposed for NoV-host interaction (387). In this model, transmission of a virus will be less efficient if the virulence (defined as mortality induced by the pathogen) is high, because under that scenario the susceptible host will be decimated in short time. Now mutant strains of lower virulence will have a selective advantage and will rapidly dominate. Because mortality is low, the host has a chance to recover, and the frequency of genetic resistance will increase after recovery. This genetic resistance is given by the HBGAs (section 1.1.7.2). Non-secretor individuals (*FUT2<sup>-</sup>*<sup>-</sup>) and B blood group are resistant to NV strain detected in the earliest outbreaks. Low virulent strains that were
able to recognize the basic type 1 precursor, in this resistant population could infect then, replicate and spread to other susceptible hosts. In this way new strains will dominate. This co-evolution results in subgroups that are resistant to the same strains but susceptible to others.

1.1.2.4. Genome organization

Sequence analysis reveals two clearly distinctive genome organizations for NoV and SaV (Figure 1.4) (21, 117). Both NoVs and SaVs genomes are positive-sense single stranded RNA approximately 7.7 kb in length. The NoV (as well as vesivirus) genome contains 3 separate ORFs: ORF-1 which encodes a nonstructural polyprotein; ORF-2 which encodes the major single capsid protein (VP1); and ORF-3 that encodes a small basic protein that may be a minor structural protein (VP2). The genome is 5’ protein-linked and polyadenylated at the 3’ end (258). In NoVs, the 5’ untranslated region (UTR) begins with the sequence GTG, also found at the 5’ end of the RHDV genome and occurs in FCV as GTA. This trinucleotide is part of a characteristic 4-24 nt motif that is also present at the start of ORF-2 and may play a role in replication, transcription or RNA packaging. The first initiation codon for synthesis of a large protein within a favorable context for translation is located at nt 6-22, depending on the strain (320, 409, 699). The ORF-1 encodes a large polyprotein of ~ 1700-1800 aa (depending on the strain) that is co- or post-translationally cleaved by the viral 3C-like protease (3CLpro). The six proteins encoded in ORF-1 are p48, NTPase, p22, VPg, 3CLpro, and RdRp, that show sequence motifs similar to picornavirus proteins (258). The ORF-2 is located immediately after or overlapping the 3’-end of ORF-1 by 11 (BoNoV), 17 (NV) or 20 (SV) nt creating a +1
frame shift. The single structural protein encoded, VP1, is 519-555 aa (56-60 kDa) and it can self-assemble into VLPs when expressed in the baculovirus system. The ORF-3 overlaps ORF-2 by 1-8 nt creating a -1 frame shift and returning ORF-3 to the same frame as ORF-1. This minor, basic and hydrophilic protein has a counterpart in all other caliciviruses and it may function in RNA genome packaging (210). The synthesis of VP1 and VP2 in non-enteric animal caliciviruses that can be propagated in tissue culture is initiated from a 3’coterminal subgenomic RNA (449, 698). This can not be proven for NoVs because of the lack of cell culture. However the detection of ~2.2 kb RNA in stool samples from human volunteer studies (320), in-vitro studies that show increasing amounts of subgenomic RNA after transfection of cells with a cDNA clone of NV (19) and increasing amounts of subgenomic RNA in MNV-1-infected cells (698), suggest that NoVs, like other RNA viruses, regulate the synthesis of structural proteins by subgenomic RNAs.

On the other hand, Sapovirus (as well as lagovirus and the proposed becovirus) genome consists of two ORFs: ORF-1 encodes a nonstructural polyprotein and capsid, whereas the ORF-2, encodes a small basic protein (116, 117, 593). In SaVs, the 5’ UTR also begins with the trinucleotide GTG as part of the 12-13 nt motif, which is also present at the 5’ end of subgenomic RNA in HuSaVs (117, 256, 407). This motif is slightly shorter (9 nt) for Po/SaV/Cowden/80/US (246). The first initiation codon occurs at nt 13-14 for HuSaVs and nt 10 for PoSaV. The main difference between NoVs and SaVs resides in the ORF-1. Unlike NoVs, SaVs ORF-1 encodes both non-structural and capsid protein (VP1), where the genome encoding the capsid protein (VP1) is in the same frame and continuous with the non-structural protein forming a large polyprotein of ~ 2300 aa
(depending on the strain) (117). The ORF-2 overlaps the 3'-end of ORF-1 by 1-4 nt creating a -1 frame shift. The small (164 aa), basic and hydrophilic protein (VP2) encoded by ORF-is analogous to ORF-2 of lagoviruses and becoviruses, and the small protein encoded by ORF-3 in NoVs and vesiviruses (118, 495). Like NoVs, synthesis of VP1 and VP2 is predicted to occur from a subgenomic RNA.

A third ORF is predicted in GI, GIV and GV but not in GII and GIII represented by London and Cowden strains, respectively. The initiation codon is located 11 nt downstream from the capsid start codon in a +1 frame shift (117). In contrast to the conserved 5’ NTR, the 3’ NTR varies in length and sequence among strains and it is followed by a poly A tract.

1.1.2.5. Virus proteins and structure

The lack of a cell culture for propagation of HEC and AEC has delayed efforts to understand virus structure and protein function. However 3 major strategies have been used to overcome this. First, after the cloning of NV and many other strains, the development of recombinant expression systems and cDNA clones (19, 532); second the genomic similarities between members of different genera within the *Caliciviridae* family which have also led to same conclusions by analogy (79); and third the recent propagation of MNV-1 in cell culture. Although MNV-1 doesn’t share the same tropism as NoVs, it is genetically closer than FCV to HuNoV and confirms in cell culture suggestions from in-vitro expression studies (134, 135).

**p48 (N-term):** The N terminal protein p48, differs from other NoV proteins in that it does not show significant similarity to other picornavirus proteins. The only similar
sequence is an H box/NC motif that is suggested, but not proven to be involved in regulation of cellular proliferation and detected in parechovirus (289). Sequence differences between GI and GII NoVs decrease at the C-terminal region of p48. The hydrophobic domain in this region was able to relocate a fused reporter gene from cytoplasmic and nuclear distribution to a Golgi location. However this potential transmembrane domain was not necessary for a peri-Golgi localization of the full-length p48. Ettayebi et al 2003 identified the SNARE regulator vesicle-associated membrane protein-associated protein A (VAP-A) as a binding partner of p48. The authors observed that expression of the vesicular stomatitis virus G glycoprotein on the cell surface was inhibited when cells coexpressed p48, suggesting that p48 disrupts intracellular protein trafficking (169).

**p41 (NTPase):** The presence of three specific motifs, A, B and C also present in poliovirus (PV), classifies p41 as a RNA helicase (522). Similar to PV, NoV p41 binds and hydrolyzes adenosine triphosphate (ATP), but it is not able to unwind the RNA:DNA heteroduplex, suggesting that p41 has ATPase, but not helicase activity. Unlike in PV, NoV p41 hydrolyzes all nucleoside triphosphates (NTPs) and this activity is not inhibited by guanidine hydrochloride (522).

**p20:** Given the similar position of NoV p20 and PV 3A within the polyprotein, it is speculated that the NoV protein is important for membrane localization of the replication complex. However because the only information available about p20 is its detection as part of a stable precursor (p20-VPg-3C<sup>Pro</sup>, analogous to the 3ABCD of picornaviruses) during the proteolytic processing of the MD145-12 ORF1 polyprotein in vitro (38), much remains to be discovered about this protein.
**VPg:** VPg is ~15kDa and covalently linked through a Tyr-24 residue to genomic and subgenomic mRNA (454). The role of VPg in initiation of replication of NoVs RNA was studied in vitro using recombinant 3D\textsuperscript{RdRp} expressed in *Escherichia coli* (550). It is proposed that replication of polyadenylated genomic RNA is a protein-primed initiation mechanism, where uridylylation of VPg-protein-prime and elongation of RNA is initiated by 3D\textsuperscript{RdRp} by a similar mechanism like 3D for PV. However a different mechanism that does not include VPg appears to be involved in replication of anti-genomic or anti-subgenomic RNA (551).

The molecular weight of NoV VPg is clearly higher than PV VPg (2-4 kDa) but closer to potyvirus VPg (24-26 kDa). The latter binds to translation initiation factors (eIF), suggesting a role in RNA translation in addition to the replication activity (394). Similar in vitro experiments have demonstrated the ability of NV and Snow Mountain virus (SMV) VPg to bind eIF3, eIF4G1 and 40S ribosomal subunit, suggesting its role in recruitment of translation initiation complexes to virus RNA (134). Similar interactions were observed when RAW 264.7 cells were infected with MNV-1 (135). Whether VPg binds 40S directly or indirectly by eIF3, or whether other eIFs also directly bind VPg or are present as part of the initiation complex it is not yet clear, but the information available suggests a novel mechanism that was able to inhibit cap-dependent and internal ribosome entry site (IRES)-driven translation as well as a eIFs-independent translation mechanism (134).

**3CL\textsuperscript{pro}:** The 3CL\textsuperscript{pro} (19kDa) is a cysteine protease responsible for the cleavage of the polyprotein encoded by ORF1 into different intermediates and individual proteins in *cis* and *trans* (38, 53, 408, 580, 608). A time course analysis of proteolytic processing
of the MD145-12 ORF1 polyprotein in an in vitro coupled transcription and translation assay identified stable precursors (p20VPg; 3CL\textsuperscript{pro}3D\textsuperscript{RdRp}) and final products (N-terminal protein; NTPase; p20; VPg; 3CL\textsuperscript{pro},3D\textsuperscript{RdRp}) (38).

Studies of NoV GI Chiba and Norwalk strains, confirmed an active nucleophilic residue in the conserved GDCG motif. The protein has a functional catalytic dyad comprised of His30 and Cys139 without a carboxylate, similar to Hepatitis A (HAV), but different from PV (259, 606).

Substrate cleavage site specificities have been investigated for several NoVs. Two potential polyprotein cleavage sites containing the QG recognition site were identified at position 399 and 762 of the NTPase, and another two sites that release VPg and 3CL\textsuperscript{pro} (408). In vivo, the proteolytic processing strategy of MNV-1 during replication in cells defined five dipeptide cleavage sites: E341/G342, Q705/N706, E870/G871, E994/A995, and Q1177/G1178 (608).

3D\textsuperscript{RdRp}: The structural and functional characteristics of NoV and SaV RdRp have been studied (190, 214, 550). Both enzymes were expressed in Escherichia coli and purified. Use of X-ray crystallography shows that both display catalytic and structural elements similar to other RdRp from RNA viruses in terms of fingers, palm and thumb domains. Initiation of RNA synthesis occurs in a protein-primer dependent manner on polyadenylated substrate as described for PV, foot-and-mouth disease and also RHDV (424, 468, 516). However a difference between them and other caliciviruses is the presence of a carboxyl terminus within the active site cleft, functioning as a potential stabilization for initiation of RNA replication (470). Also a second mechanism of initiation was described for NoVs and SaVs but not for RHDV, FCV or PV. In this de novo initiation mechanism
neither primer nor protein-priming is involved and it takes place on heteropolymeric templates (190, 551).

**VP1 (major capsid protein):** This single major structural protein ranges from 530-550 aa (58-60 kDa). Expression of VP1 using a baculovirus system (319), bacterial vectors (630) or mammalian cells (633), results in self-assembly of the virus protein into virus-like particles (VLPs) with a morphology similar to the native virus. The VP1 of SaVs differs from NoVs in that the capsid protein is encoded in ORF1. An upstream sequence of 73 nt was necessary for production of the capsid protein in a baculovirus expression system maybe due to the distinct genome organization (322). The structure of these VLPs was resolved by electron cryomicroscopy, computer imaging processing and X-ray crystallographic methods as a T = 3 icosahedral symmetry (531, 533). The VP1 folds into 90 dimers that compromise, from the N to C terminal, the shell domain (S) (aa 1 to 225) and a protruding domain (P) that is then subdivided into a central stem domain P1A (aa 226 to 278), a globular domain P2 (aa 279 to 405), and P1B (aa 406 to 520), with P2 at the most distal surface of the VLPs and directly involved in virus attachment (Figure 1.5) (629). The S-domain is essential for assembly of the icosahedron, whereas P-domains interact in dimeric contacts that stabilize the capsid and form the protrusions on the virions (531). Overall, the icosahedral and subunit domain organization is similar for NoVs, SaVs and SMSV (108, 109). However, significant structural variations among different strains were observed in the P-domain (particularly P2), but not in the S-domain. The P2-domain (127 aa) contains a hypervariable region (or binding pocket) that plays a role in receptor binding and immunity, and is responsible for ABO-histo-blood group antigen interactions associated with susceptibility to infection (629).
**VP2 (minor capsid protein):** This protein is encoded by the ORF-3 in NoVs and ORF-2 in SaVs. It is 208-268 aa (~22-29kDa) long and exhibits high sequence variability among strains (579). Different roles during the replication cycle have been proposed (210). The high Isoelectric point (pI), its basic nature, and its chemistry led to the conclusion that it might function as an RNA binding protein, perhaps involved in RNA genome packing (211). It also has a regulatory function for expression of VP1 and the stability of the virus particles (44). The VP2 as well as VP1 are translated from a subgenomic RNA. The mechanism of expression of VP2 is particularly dependent on the termination codon for VP1 and 84 nt preceding VP2 sequence (448). A mechanism that regulates synthesis of VP2 relative to VP1 was also suggested based on the observation that the initiation codon for VP2 is in a poor sequence context for translation (448).

### 1.1.2.6. Physicochemical and biological characteristics

Enteric viruses are acid stable and can survive in the gastrointestinal tract. Most viruses remain infectious after refrigeration and freezing and they also retain their infectivity after heating. Norwalk virus remains infectious after exposure to pH 2.7 for 3 h and after heating at 60°C for 30 min as demonstrated by human volunteer studies (149). However NV VLPs have shown relatively less stability (pH: 3-7 and up to 55°C) (25), suggesting that the medium composition could positively influence virus survival. Enteric caliciviruses, with the exception of PoSaV/Cowden strain (248), can not be routinely propagated in the laboratory. Recently a 3D model, using aggregates of highly differentiated intestinal epithelial cells was established as in vitro cell culture model for HuNoVs (615). However, although successful virus infections was demonstrated, the
level of virus replication that could be achieved with this system is under investigation (100). Because of this, most of the attempts to determine biological properties and methods that can inactivate calicivirus based on these characteristics have been done with FCV and canine calicivirus (CaCV), both members of the Vesivirus genus and already adapted to cell culture, although results can not be completely extrapolated given the structural and stability differences between FCV, CaCV and ECV.

**Chlorine:** Chlorine–based disinfectants are considered the most effective against enteric viruses. However caliciviruses appear to be relatively resistant to chlorine. Noroviruses are resistant to inactivation with 3.75 mg/ml-6.25 mg/ml chlorine which is the amount of chlorine in drinking water in the U.S. (348), but they are inactivated by 10 mg/l which is the amount used to treat a water supply after an outbreak (149). Inactivation of FCV in surfaces, as surrogate of NoVs, was achieved with 3,000ppm (or higher) for 10 or 30 min at room temperature (156). In another study, FCV (as a surrogate for NoV) and *E.Coli* were inactivated by >4 \log_{10} within 5 min with a dose of 30 mg/l of applied chlorine (645).

**Temperature:** Several studies have been done to determine heat inactivation of caliciviruses and the investigators concluded that the time required for inactivation increases as the incubation temperature decreases (9, 156, 327, 592). Norwalk virus remains infectious after heating at 60ºC for 30 min as demonstrated by human volunteer’s studies. Again by using FCV as surrogate, Allwood et al 2003, compared the survival rates of FCV at 4ºC, 25ºC and 37ºC for up to 28 days in dechlorinated water, showing that 90% reduction in infectious titer is achieved at 3 days at 37ºC, but the value increased to 5.2 days at 25ºC and 7.3 days at 4ºC (9). Similar results were obtained in
marine water at 4°C and 10 °C (327) or seawater where an initial 20-fold decrease in infectivity occurred upon its addition to seawater, but no significant decrease occurred during the next 24 h (592). Virus inactivation was achieved in 1 min at pasteurization temperature (71.3°C), 8 min at 56°C, 24 h at 37°C but it required 1 week at 20°C (156).

**U.V. irradiation:** Inactivation by U.V. is considered particularly effective on viruses with large or single stranded genomes. However results differ when a different source (shape, size and lamp geometry) of light or protein solution composition are assayed (672). In a first study, infectivity reduction of 90% was obtained with U.V. dose of 480 J/m² for FCV, 120 J/m² more than the dose required for HAV and double the amount required for 90% inactivation of PV (481). In a second study, only 60 J/m² were required to achieve the same reduction in buffered demand-free water (639). In another study, De Roda Husman and colleagues (2004) not only compared the rate reduction by U.V. for FCV (respiratory) and CaCV (enteric), concluding that both viruses required similar U.V. doses for inactivation Because capsid sequence homology is only 37% between FCV and CaCV, the authors proposed that these two viruses can be used as surrogates for NoVs because differences in capsid sequence did not result in differences in sensitivity to U.V. (139).

**pH:** Norwalk virus retains its infectivity after exposure to pH lower than three for 3 h at room temperature (149). Although a correlation was found between the decline of animal calicivirus RNA detection (FCV and CaCV) and the decline of NoV RNA detection for different treatments, the capsid stability differed for each virus after exposure to different pHs. Whereas DenHaag22 strain (a HuNoV GII/4) RNA was protected after exposure to high and low pHs (pH 2, 3, 10 and 12) for 30 min at 37°C,
FCV and CaCV RNAs were degraded at pH lower than 3 and higher than 10, suggesting that the NoV capsid has a higher stability (156). Organic acids are unlikely to have any effect on the viability of these viruses during short contact times (582).

**Other inactivation methods:** Inactivation by several other commonly used disinfectants has been assayed. A first study showed that whereas glutaraldehyde and iodine-based products effectively inactivated different suspensions of FCV, quaternary ammonium products, anionic detergents and 75% ethanol (v/v) failed to inactivate it (152). A second study on steel surfaces indicates that among seven commercial disinfectants for surface and three sanitizers for fruit and vegetables in one, two and four times the manufacturer’s recommended concentrations for contact times of 1 and 10 min, only the phenolic compounds and a combination of quaternary ammonium compound and sodium carbonate at twice the recommended concentrations inactivated FCV (241).

A different approach considered is the capacity of ozone as an oxidant and its effectiveness as a disinfectant against most waterborne pathogens (602). Overall, the results of this study indicated that NV as well as other enteric viruses can be reduced rapidly and extensively by ozone disinfection in water (588). As a follow-up, Hudson et al 2007, demonstrated that ozone gas inactivated FCV (as determine by infectivity assay) in dried samples placed at different locations in closed environments, such as offices, hotel rooms or cruise ship cabins and correlated this result with the ones obtained for NV RNA in offices (288).
1.1.3. REPLICATION

Single stranded, positive sense RNA (ssRNA+) viruses (except the retroviruses) replicate in the cytoplasm. Viruses can be divided into two groups based on their replication strategy: those that produce subgenomic RNAs and those that do not. Caliciviruses belong to the first group, where genomic RNA directs the translation of a polyprotein precursor that includes only non-structural proteins, including the RdRp. These proteins will later catalyze: 1) RNA replication through the synthesis of full length anti-genomic RNA; 2) synthesis of virus structural proteins by synthesis of one or more subgenomic RNAs from which the structural proteins will be translated. The replicated genomes will be translated to produce more non-structural proteins and assembled with structural proteins into progeny viruses (Figure 1.6) (29). However each family displays different strategies to pursue the various steps. In the case of HECV and AECV, the lack of a routine cell culture system where these viruses can be propagated has delayed an understanding of virus replication and the development of control strategies. Several concepts have been learned from information about other (non-enteric) caliciviruses, such as FCV that can be fully propagated routinely in cell culture. Also in recent years, information have been obtained through expression studies with infectious clones, recombinant proteins and the discovery of MNV-1, which although it is not an AECV, it is closely related to HuNoVs and can replicate in cultured macrophages and dendritic cells.
1.1.3.1. The replication cycle

Replication of calicivirus occurs on the surface of membranous vesicles in the cytoplasm of the infected cell. Attachment and penetration is receptor mediated by one or more molecules present on the surface of the target cell. In the case of FCV, the Junction adhesion molecule 1 (JAM-1), present in tight junctions between Crandell-Rees feline kidney (CRFK) cells, as well as alpha 2,6-linked sialic acid residues present on an N-linked glycoprotein on the surface of Vero cells were identified as the functional receptors for FCV (428, 618). Entry is dependent on clathrin-mediated endocytosis and acidification in endosomes (619). Noroviruses recognize human histo-blood group antigens (HBGAs) as receptors. The recognition of human HBGAs by NoVs is a protein-carbohydrate interaction, in which the P domain of the NoV capsid (or VLPs) binds to the oligosaccharide side-chains of the HBGAs (628, 629). This has also been observed in tissues of oysters where NoVs bind carbohydrate structures with a terminal N-acetylgalactosamine residue, similar to the one present in HBGAs (382, 640). Different NoV strains have been demonstrated to recognize and bind to different HBGAs present on enterocytes leading to at least 7 receptor-binding patterns among 14 NoVs strains (287, 387, 629). However the entry mechanism is unknown due to lack of a routine cell culture system.

After uncoating, successful translation of the polyprotein precursor requires a mechanism for subverting the host cell translation machine benefit the virus. To achieve this, picornaviruses for example, cleave the eIF4G by their protease, resulting in disassembly of the 43S initial replication complex required by host capped mRNA. However, picornavirus RNA is still translated because of the IRES in the 5’ UTR of the
virus genome that drives a cap-independent translation mechanism (39). Unlike other ssRNA+ viruses, caliciviruses do not have a 5’cap structure or IRES to initiate direct translation of their RNA and they require the interaction with eIFs. Instead NoVs, vesiviruses and lagoviruses initial translation depends on the VPg protein covalently linked to the 5’end of the viral genome, and latter linked to subgenomic RNA for synthesis of structural proteins (258). In vitro experiments with purified eIF3 and eIF3 in mammalian cell lysates demonstrated that NV and SMV VPg bind eIF3, and directly or indirectly through their interaction with eIF3, they also bind eIF4G1 and 40S ribosomal subunit (134). The same interactions were observed \textit{in vitro} and in cells (RAW 264.7 cells) infected with MNV-1 (135). This last experiment also proves that MNV-1 VPg co-precipitates eIF4E directly or indirectly bound to eIF4GI. The same eIF4GI-4E interaction was essential for FCV but not for MNV-1 replication, whereas both required the presence of eIF4A (helicase component) (105, 215). All this information suggests a novel mechanism for animal RNA virus translation common to Vesiviruses and NoVs, but with different eIF requirements among them and similar to the mechanism proposed for members of the \textit{Potyviridae} family (394).

In vitro proteolytic hydrolysis of the polyprotein precursor is mediated by the virus protease and release a group of stable intermediate complexes and final products in a time frame process. For NoVs, particularly MD145-12 strain (HuNoVs GII.4), in vitro coupled transcription and translation assays showed that co-translational cleavage of two QG dipeptides (Q^{330}/G^{331} \text { AND } Q^{696}/G^{697}) results in release of the N-terminal, NTPase and a p20VPgProRdRP complex. Two pathways were proposed for processing of this complex. In one of them, the cleavage of E^{1008}/A^{1009} releases p20VPg and ProRdRp,
which accumulates in the cell (38, 605). In the second pathway, the p20VPgPro is released from the RdRp by cleavage of $E^{1189}G^{1190}$ and two further cis cleavages by Pro releases p20 from VPgPro and finally VPg from Pro (38). The p20VPg and ProRdRp complexes detected for NoVs are similar to those detected during in vitro or cell culture studies of other HuNoVs (53), MNV-1 (608), FCV (610) and RHDV (362, 450), suggesting that the mechanism is similar and some of these intermediates are important for the virus replication strategy. One difference between the NoVs and other caliciviruses is the absence of an N-termNTPase intermediate, that could be a consequence of the high efficiency of NoVs Pro (259, 408) compared with other caliciviruses.

Replication of viral RNA occurs in replication complexes associated with cell membranes. Formation of this complex requires a network of protein-protein interactions between the virus and the host cell and results in reorganization of intracellular membranes and sometimes the loss of the Golgi complex as observed for HuNoVs and MNV-1, but not for FCV (177, 233, 330, 698). Enzymatically active replication complexes have been isolated from FCV infected cells (233). Non-structural protein precursors such as p76 (NoV Pro-RdRp) and p30-VPg (NoV p20-VPg), as well as the mature p32 (N-term), p30 (p20) and p39 (NTPase) were detected in the complex. Increasing amounts of VP1, simultaneously with increasing amounts of a 2.2 kb antigenomic RNA were observed. Several protein-protein interactions in this complex could be associated with RNA replication and translation. Interactions between FCV p32 (analogous to NoV p48 or N-term) with the viral NTPase, p30 (NoV p20) and the ProRdRp complex were identified suggesting its possible role as an integral membrane
protein that anchors many of the other components in place (330). A similar function was suggested for HuNoV (NV and MD142-12) N-term resulting in disassembly of the Golgi complex in transfected cells (177). Interactions detected between p76 and VPg, could be involved in RNA replication since VPg is linked to genomic and subgenomic RNA, p76 retains the polymerase activity and possesses the ability to transfer nucleotide to VPg, suggesting a protein-primer mechanism of RNA synthesis (424).

A novel mechanism for initiation of replication by NoV RdRp was proposed. In 2004, Fukushi et al, used a recombinant baculovirus system to express enzymatically active RdRp from NV genome and to determine the conditions for RNA replication using an RNA template from the NV 3’ genome region. The authors observed similar levels of activity with or without a poly(A) tail, or after the addition of an RNA primer, concluding that the RdRp exhibits a primer- and poly(A) independent RNA polymerase activity (189). Also Belliot et al (37) observed this fact, demonstrating that both ProRdRp and RdRp of MD145-12 (NoV GII.4) are active forms of the polymerase and suggesting a copy-back mechanism for initiation of replication, as previously suggested for RHDV and FCV RdRp (417, 681). In 2006, Rohayem et al demonstrated that NV RdRp yields two different products when incubated in vitro with synthetic RNA: 1) a double stranded RNA (dsRNA) consisting of two single strands of opposite polarity; and 2) a ssRNA template labeled at its 3’ end by the RdRp terminal transferase activity. Unlike previous studies, the authors suggested that RNA synthesis occurs de novo (550). In a follow up study (551), the same authors concluded that two different mechanisms for RNA replication are executed by the NV RdRp. Initiation of RNA synthesis on homopolymeric templates as well as replication of subgenomic polyadenylated RNA is accomplished in a
proteins-primer dependent mechanism that involves elongation of VPg by RdRp, similar to the mechanism described for PV (516). In contrast, replication of anti-subgenomic RNA was not primer dependent, nor did it depend on a leader sequence on the 3’ end. In this case, when the template is nonpolyadenylated, the RdRp initiates de novo synthesis and terminates RNA synthesis by a poly (C) stretch. Moreover this poly (C) RNA will be the template for de novo synthesis in the presence of high concentrations of GTP (551). The same mechanism was described by Fullerton et al in 2007 for SaV RdRp.

Structural proteins are next translated from VPg linked subgenomic RNA. It was observed that Vp2 is translated as 10-20% of VP1. How this occurs is unclear. The 3’ end of NV mRNA contains determinants that regulate the expression and stability of VP1. Comparisons of the kinetics of RNA and capsid protein expression levels by using constructs with or without ORF3 or the 3'UTR revealed that the 3'UTR increased the levels of VP1 RNA, whereas the presence of VP2 resulted in increased levels of VP1. Furthermore, VP2 increased VP1 stability and protected VP1 from disassembly and protease degradation (44). Interestingly, in the case of lagoviruses, where VP1 is encoded by the ORF1 (like the non-structural proteins) and VP2 is encoded by ORF2, translation of VP2 is apparently regulated by the 3’ end sequence of ORF1, in a process of termination/reinitiation that is independent of an AUG (448).

Studies by Asanaka et al (2005) with a NV infectious clone demonstrate that genomic RNA was packaged when it was co-expressed with subgenomic RNA, whereas subgenomic RNA alone was not packaged when expressed alone, suggesting that RNA packaging signals may reside in the ORF1 and non-structural proteins may be required.
for packaging (19). It is also suggested that because of its basic nature, VP2 could bind RNA and function during genome packaging (210).

1.1.3.2. Effect on host cells

Infection of host cells or cell cultures, when available, causes rapid cytopathic effects followed by cell death. The physiological mechanisms associated with this are reviewed in this section. Macro and microscopic histological changes are discussed in the pathogenesis section (1.1.4).

Apoptosis, is a controlled mechanism involved in development, tissue homeostasis, and elimination of damaged cells, but it can also be targeted by virus to replicate and spread or to avoid the host’s immune response (634). Induction of apoptosis in CFRK cells infected with FCV was defined by characteristic changes such as translocation of phosphatidylserine to the cell outer membrane, chromatin condensation and oligonucleosomal DNA fragmentation, and increasing levels of caspase-3, -8 and -9 (544, 609). Rabbit hemorrhagic disease is a rapidly lethal disseminated infection characterized by acute liver damage. Similar to FCV, Alonso et al 1998, found that liver cell death induced upon infection with RHDV was due to apoptosis (10). A few years latter, immunohistochemical labeling for RHDV antigen-positive cells, and TUNEL assay for apoptotic cells in hepatocytes collected from RHDV infected rabbits confirmed these results (326).

In the case of NoVs, increased numbers of apoptotic cells by the TUNEL assay were detected in gnotobiotic (Gn) pigs inoculated with HuNoV HS66 strain (NoV GII.4) when compared to the control pig tissues (106). Clinical cases of allograft lesions from
pediatric intestinal transplant patients were common to allograft rejection, but increased superficial apoptosis was characteristic of HuNoV enteritis (462).

### 1.1.3.3. Infectious clones and cell culture

Studies of virus replication are based on cell culture systems. However, the main challenge in ECV studies is the fact that, despite numerous attempts, NoVs and SaVs cannot be routinely propagated in cell culture (157), with the exception of PoSaV/Cowden strain and MNV-1. For HuNoVs, more than 25 primary culture and cell lines, on solid or permeable surfaces, with different cell culture supplements were assayed (157). Different NoVs strains within GI and GII, different methods for virus preparation, cell inoculation, and conditions for maintenance of cell monolayers, were also assayed without success. Binding of NV VLPs to differentiated Caco-2 cells was demonstrated by competitive experiments. However only 1.4 to 6.8% of prebound VLPs were internalized into cells (684).

Recently NoVs were shown to infect a 3-dimensional (3-D), organoid model of human small intestinal epithelial cells (INT-407) (615). The model shows that NoVs will replicate (as detected by microscopy, reverse transcription-polymerase chain reaction (RT PCR) and fluorescent in situ hybridization), if the appropriate cells (INT-407) are grown as 3D aggregates and they are infected when they are fully differentiated (615). The authors hypothesized that the success is not only because of the physiology of a 3D model but also the “co-culture” of multiple intestinal cell types (enterocytes, goblet cells, and M-cell like markers), that has been hypothesized as a factor likely needed for NoV infectivity (157).
The PoSaV/Cowden strain was first adapted to serial propagation in primary porcine kidney (PPK) cell cultures (182). Different intestinal enzymes, pH conditions and also non-supplemented medium were assayed. However, successful propagation was only achieved by adding intestinal content preparation (IC), derived from uninfected Gn pigs to cell culture. Thereafter, PoSaV/Cowden was propagated in two continuous porcine kidney cell lines (LLC-PK and PK-15 derived from epithelial-like cells in the proximal renal tubule. Again, attempts to propagate the virus in the presence of different intestinal enzymes of swine origin, bacterial protease, or IC from other species were unsuccessful, and the virus infects cells and propagates only in the presence of IC from uninfected Gn pigs (510).

The tissue culture-adapted PoSaV/Cowden (TC-PoSaV/Cowden) shows one silent mutation in its protease (T1221), two amino acid changes (Y1252 to H, and R1379 to K) and a silent mutation (N1263) in its RdRp, and five nucleotide substitutions in its capsid that result in one distant (C178 to S) and three clustered aa changes (Y289 to H, N291 to D and K295 to R) and a silent mutation (T336) compared with the wild-type PoSaV/Cowden (WT-PoSaV/Cowden). The TC-PoSaV/Cowden doesn’t cause diarrhea in orally (PO) inoculated Gn pigs whereas Gn pigs inoculated with WT-PoSaV/Cowden developed diarrhea, lasting for 2 to 5 days (248). In an attempt to identify the specific factor in IC promoting virus growth, Chang et al (101) demonstrated that the IC-promoting effects on virus growth were on cells rather than on the virus. The authors also demonstrated that replication of PoSaV/Cowden may initially depend on a cyclic AMP (cAMP) signaling pathway induced by IC because inhibitors of cAMP inhibited the effect of IC in a dose-dependent manner for up to 72hs (101). In 2004, bile acids were
identified by Chang et al (103), as active factors in IC essential for growth of PoSaV/Cowden in cell culture. Several commercially available bile acids supported virus replication and induced increased levels of intracellular cAMP in LLC-PK cells. Moreover, individual bile acids induced higher levels of cAMP than IC. Bile acids activate the Protein Kinase A (PKA) signaling pathway, because addition of different inhibitors of this pathway blocks the bile acid mediated-PoSaV growth. The authors also demonstrated that bile acids could down-regulate Interferon (IFN) mediated by signal transducer and activator of transcription 1 (STAT-1) activation. However in this case, the IC showed higher levels of activity than individual bile acids. Given the essential role of STAT-1 for antiviral innate immunity by IFNs, the authors proposed that its down regulation by bile acids might allow PoSaV/Cowden replication in vitro and in vivo (103).

The MNV-1 can be grown in primary cultures of macrophages and dendritic cells and RAW cells (a macrophage derived cell line) (698). Although the cell/tissue tropism of MNV-1 (NoV GV) differs from the HuNoV strains, this system is useful to study the replication of a NoV. Like TC-PoSaV/Cowden, serial passage of MNV-1 in cell culture resulted in two silent mutations and three aa changes located at the 3A protein (H845R,V716I) and the hypervariable P2 domain (K5941Q) similar to TC-PoSaV/Cowden. Same as TC-PoSaV/Cowden the cell culture adapted MNV-1 was attenuated when inoculated in STAT-1-/- mice MNV-1 strain (698).

In an attempt to overcome this barrier, meaning the lack of a cell culture to study replication of AECV and HECV, different infectious clones have been developed (19, 102, 104, 340). In 2005, Chang et al developed an infectious clone for PoSaV/Cowden strain (102). A full-length cDNA copy of PoSaV/Cowden genome was cloned into a
plasmid vector downstream from the T7 RNA polymerase promoter, and capped RNA transcripts were infectious when transfected into LLC-PK cells. Recovery of PoSaV depended on the presence of bile acids and the recovered particles induced mild diarrhea when PO inoculated in Gn pigs like as the TC-PoSaV/Cowden

The first infectious clone for NV was developed by Asanaka et al in 2005. Replication of NV RNA and packaging into virus particles was achieved in human embryonic kidney (HEK) 293T cells by intracellular expression of NV RNA derived from an expression vector using a replication-deficient vaccinia virus MVA encoding the bacteriophage T7RNA polymerase (MVA/T7) (703). The presence of subgenomic RNA transcribed from genomic RNA by the non-structural proteins translated from the genomic RNA and further translation into VP1 was demonstrated. Packaging of the viral genomic RNA into virus particles was achieved when subgenomic RNA was coexpressed, but not when subgenomic RNA was expressed alone, indicating that packaging signals may belong to ORF-1 (19). One year latter a full length cDNA infectious clone was engineered for a NoV GII strain and expressed in 293T/17 cells using the vaccinia virus expression system (340). However in this case, although genomic, anti-genomic and subgenomic RNA were detected, viral proteins were detected only when subgenomic RNA was co-transfected.

Recently, the generation of cells expressing self-replicating NV RNA (NV replicon) was developed (104). Expression of replicon RNA was achieved by transfection of engineered neomycin resistant-NV RNA into human hepatoma (Huh-7) or baby hamster kidney (BHK21) cells. Although the replicon is not able to produce virus particles, because the VP1-encoding region was disrupted by introducing a neomycin
resistant gene, the presence of genomic, anti-genomic RNA as well as non-structural proteins corroborates replication. A similar replicon system allowed the identification of evasion mechanisms of intracellular host defense by Hepatitis C virus (HCV) (192).

1.1.4. DISEASE AND PATHOGENESIS IN NATURAL AND EXPERIMENTAL MODELS

1.1.4.1. Human infection

Norovirus infection is characterized by the sudden onset of vomiting or diarrhea or both, symptoms that vary from person to person according to human volunteer studies (150, 223). Nausea, abdominal pain, abdominal cramps, anorexia and malaise also occur. Diarrhea is non bloody, lacks mucous and may be loose and watery (644). Headache, fever, chills and myalgias occur in 25-50% of infected people and approximately 50% develop low fever.

The average incubation time is 12-48 h and symptoms usually resolve in 12-72h according to data from volunteers studies and outbreaks (223). However, recently studies have described longer periods of illness. In The Netherlands, the illness extended from 6 days in children under 1 years of age versus 4-5 days in 1-11-year-old children versus less than 3 days in children 12 or more years of age (546). Hospitalized patients have a longer median duration of illness of 3 days and 40% of patient older than 80 years old remained symptomatic after 4 days (422). Infection is self-limiting and patients usually recover without sequels. However asymptomatic shedding of virus may exceed 22 days (546), contributing to transmission of disease. Risk groups for clinical complications are
those who can not maintain hydration because of a preexisting condition, those immunocompromised or under significant environmental stress (89, 345).

The pathogenesis of HuNoVs has been described in human volunteers and in animal models. Broadening and blunting of intestinal villi at the duodenojejunal junction, crypt cell hyperplasia, cytoplasmic vacuolization and infiltration of inflammatory cells into the lamina propria were observed in biopsies from infected volunteers (6, 570). These histological changes have been seen in symptomatic and asymptomatic persons as well as in a symptomatic patient with small intestine transplant (462). Histopathologic changes resolve in 2 weeks in healthy subjects. The mechanism of virus-induced diarrhea and vomiting is unknown (643).

A Gn pig model was developed to study the pathogenesis of HuNoVs (106). The rationale behind this model resides in the fact that pigs resemble humans in their gastrointestinal anatomy, physiology and immune responses and PoNoVs genetically similar to HuNoVs are infectious for Gn pigs (563, 675). Seventy-four percent of the inoculated animals developed mild diarrhea and NoV RNA or antigen were detected in rectal swab fluids and intestinal contents (IC). Mild lesions were detected in the proximal small intestine of one pig. Immunofluorescence revealed the presence of VP1 in duodenal and jejunal enterocytes, as well as non-structural proteins that confirmed translation. Cytoplasmic membrane vesicles containing calicivirus-like particles were detected by transmission electron microscopy (106).

Sequential passage of Toronto strain (NoVs GII) was achieved in Macaca nemestrina. Clinical illness characterized by diarrhea, vomiting and dehydration occurred in inoculated monkeys. Presence of the virus up to 21 days in stool samples was
confirmed by RTPCR and enzyme linked immunoabsorbent assay (ELISA) and infectivity of these samples was demonstrated by transmission from infant to their parent in contact with them (620). Susceptibility to NV (NoV GI) and Grimsby (Nov GII) strains was also tested in common marmosets, cotton top tamarins, *cynomolgus macaques* and *rhesus macaques* (547). None of the animals showed clinical signs and only 1/4 *rhesus macaques* showed virus shedding for up to 19 days (by RTPCR) with specific IgG and IgM antibody responses at week 2 post-inoculation. This information indicates that *rhesus macaques* are susceptible to infection by NV strain but more studies are required to define the model.

1.1.4.2. **Animal infection**

**Porcine Norovirus and Sapovirus**

The WT-PoSaV/Cowden strain induced intestinal lesions in PO or intravenously (IV) inoculated Gn pigs similar to those seen in HuNoVs (183, 248). Moderate to severe villous atrophy and fusion, crypt cell hyperplasia and reduction of villous crypt ratios, vacuolization, and infiltration of polymorphonuclear and mononuclear cells into the lamina propria were observed in the duodenum and jejunum, but only mild or no villous atrophy was seen in the ileum. PoSaV-infected enterocytes of duodenum and jejunum were detected by IF, but the crypt enterocytes were PoSaV-negative. These findings indicate that PoSaV, like HuNoVs, mainly infects villous enterocytes and induces lesions in the proximal small intestine of infected Gn pigs.

Porcine noroviruses were isolated from healthy finisher pigs in the U.S. Limited information is available on its pathogenesis. Inoculated Gn pigs seroconverted and virus
RNA was detected by RT-PCR (675). One pig inoculated with Po/NoV/OH-QW144/03/OH showed mild diarrhea and virus shedding from post-inoculation day (PID) 3 to 5, whereas IC collected at euthanasia on PID 5 contained NoV-like particles detected by IEM. Another pig inoculated with Po/NoV/OH-QW126/03/US did not have diarrhea, but RTPCR results were positive on PID 5.

**Bovine Norovirus and Becovirus**

In Gn calves, BoNoVs (represented by Bo/NoV/Newbury2/76/UK) and members of the potentially new *Becovirus* genus (Bo/Beco/Newbury1/76/UK and Bo/Beco/Nebraska/80/US) produce similar clinical signs and intestinal lesions, but overall changes appear to be more severe in the case of becoviruses (70, 593). Histopathologic changes are limited to the small intestine where the most severe lesions were detected at the duodenum and jejunum. Villous atrophy, coupled with exfoliation and loss of villous enterocytes and crypt hyperplasia were observed (593).

**1.1.5. DIAGNOSIS**

To assess the real impact of a pathogen in human disease requires a sensitive and reliable detection method for clinical samples. The method has to be able not only to identify and differentiate the pathogen from others present in the samples, but also it should be able to determine if the detected pathogen is infectious. In the case of ECV the first problem is that NoVs and SaVs (with the exception of PoSaV/Cowden strain) do not grow in cell culture; thus detection methods rely on traditional techniques that detect either antigens, such as ELISA or molecular techniques that detect RNA, such as
RTPCR. A positive result with either of these techniques does not provide information about virus infectivity. Both antigens and RNA will eventually be degraded in the environment, but it is unknown how long this will take, since it has been demonstrated that rapid and total degradation of RNA after the particle is broken doesn’t occur (592), and also inactivation may result only in loss of capsid function, but the RNA inside remains intact and the inactivated particle could still be detected by antibodies as demonstrated for HAV and FCV (480).

Noroviruses and sapoviruses can be detected by ELISA, EM and/or RTPCR in feces of infected humans or animals (195, 248, 321, 482). Usually when the techniques are compared, RTPCR has the highest sensitivity followed by IEM and ELISA, but specificity is highest for IEM followed by ELISA and RTPCR that usually requires confirmation by another technique such as hybridization or sequencing (534). Despite these differences, all three methods will be useful for epidemiological investigations in gastroenteritis outbreaks, but for individual cases, at least two of these methods should be combined (534).

The picture becomes more problematic when considering that most of gastroenteritis outbreaks in the case of ECV are actually food or water-borne outbreaks. In this case, epidemiologic studies are required, not only to detect the virus, but also to trace it back to the source. Virus detection in water and food presents two more challenges: the complexities of food matrices and the very low infectious dose (10-100 particles) in a quantity likely to be eaten or drunk as a single portion. However, today RTPCR is the primary assay for detection of NoVs and SaVs due to its high sensitivity. In recent years, real time RTPCR (rt-RTPCR) has emerged as a potentially important
diagnostic procedure (283) as well as the nucleic acid sequence-based amplification (NASBA) technique (312, 512).

One drawback of these PCR-based methods is the unexpected high strain variability, particularly in NoVs, that makes it impossible to detect all the strains with one set of primers or one antiserum. The development of microarray technologies offers the opportunity to use several primers simultaneously to detect and classify known or unknown groups of pathogens (virus, bacteria and parasite) present in a sample in only one assay.

1.1.5.1. Antigen detection: classical techniques

1.1.5.1.1. EM and IEM

The first human NoV and SaV strains (NV and SV strains, respectively) were detected in fecal samples by EM (113, 336). The main advantage of this technique resides in the ability to detect single or mixed infections in one assay. The first PoSaV was detected in fecal samples from pigs that had a mixed infection with rotavirus (559). Although SaVs have the characteristic “Star of David” appearance from which caliciviruses get their name, NoVs do not have the typical calicivirus morphology (148). Low sensitivity (10^5-6 particles/ml) and labor-intensive are two weak points in use of EM techniques to detect caliciviruses. Underestimated prevalence due to technique sensitivity remains a problem using EM. A 6 year retrospective study performed in 1990 in 10 EM centers in the U.S. and Canada demonstrated the presence of virus only in 16% of the samples, which was very low considering that all samples belonged to clinically diagnosed gastroenteritis. The SRSVs were the third most commonly observed viruses
(after rotavirus and adenovirus) with 0-40% incidence and the second most important at 2/10 centers (398). When EM was applied to detect virus particles in stool samples from 223 outbreaks caused by NoVs in northwest England, virus particles were observed in 56% of the outbreaks (127/223) by EM (195). Nevertheless, EM is still considered the gold standard method for virus detection (363). Immune electron microscopy is a modification of EM that uses an immune serum (hyperimmune or convalescent) against a specific virus. The first studies by IEM used convalescent serum from volunteers that orally received a filtrate from one of the specimens collected during one outbreak (151). This not only allowed increased sensitivity, but also demonstrated antigenic differences between morphologically similar strains detected in U.S and Japan (151, 492). In their study of the pathogenesis of PoSaV/Cowden strain in Gn pigs, Guo and colleagues (248) detected PoSaV in feces by IEM using a hyperimmune antiserum to this strain. In another study, PoNoVs were detected in feces of experimentally infected Gn pigs by IEM using convalescent serum from a Gn pig inoculated with PoNoVs (675). Although the test sensitivity may be increased ten-fold in comparison to EM, application of IEM is still limited to samples collected during the peak of shedding (early during diarrhea) and it may also be limited by the lack of a broadly reactive antiserum that can recognize several HECV or AECV.

A modification to IEM is solid-phase immune electron microscopy (SPIEM) where broad- or virus- specific antibodies are used to coat the grids (directly or through protein A) before the sample is applied (21). The technique has been used for detection and classification of NoVs (401). Another modification of IEM used a colloidal gold-protein A conjugate to label clumps of virus and antibody in suspension (403).
1.1.5.1.2. **ELISA**

A new era in diagnostics tests began with the cloning of NV, expression of its capsid in a baculovirus expression system and production of VLPs (319). Hyperimmune serum from animals inoculated with NV-VLPs has high antibody titers to the homologous strain and it has been used to detect homologous VLPs (10^6 VLP/well) and native virus in stool samples in an ELISA format (223, 319). Since then, VLPs have been expressed for other HuNoVs and BoNoVs (147, 232, 252, 254, 318, 359, 360, 393, 399) and for HuSaVs and PoSaVs (249, 322). Because the ELISA technique allows testing of numerous samples in a short time, without any specialized equipment, ELISAs have been developed and used for large-scale epidemiologic studies of HuNoVs and HuSaVs (195, 275, 400, 466, 482) and PoSaVs. The ELISA for detection of NV is highly sensitive (0.025 ng of capsid) and specific, detecting antigen in volunteer’s stools diluted as much as 1:10,000 (223). The high sensitivity of ELISA, sometimes comparable to RTPCR is because of the presence of a soluble protein in stools of infected people (235). This soluble protein was characterized as a 32 kDa protein produced by specific cleavage of the capsid *in vivo*, although its presence or absence can not be related to infectivity (235, 261). In an attempt to compare ELISA and EM sensitivity, Gallimore and colleagues (195) studied 156 outbreaks of gastroenteritis suspected or confirmed by EM to be caused by NoVs. The 46% (71/156) of these outbreaks were positive by EM, but the results also showed that EM underestimated the number of outbreaks caused by NoVs, since 26/85 (31%) EM-negative outbreaks were positive by ELISA for Grimsby virus (a HuNoV GII). Moreover, a major weakness of ELISAs is the high specificity that each antiserum has shown for the homologous strain used to produce it, but not for heterologous strains
This is a disadvantage for screening samples in epidemiologic studies because the circulating strains are often highly diverse. Some of these weaknesses have been overcome by using pools of monoclonal antibodies (mAbs) or pool of antibodies prepared from animals immunized with multiple GI and GII NoVs and SaVs strains (21, 321, 354).

Three ELISA kits have been commercialized. Two of them, IDEIA® NLV (Dako Cytomation Ltd., Ely, United Kingdom) and RIDASCREEN® norovirus EIA (R-Biopharm AG, Darmstadt, Germany), although they are not licensed in the U.S., are available worldwide and were evaluated against stool samples containing different HuNoV genotypes within GI and GII and HuSaVs. Although the results obtained differed among studies in terms of kit sensitivity and cross-reactivity between HuNoVs GI and GII and HuSaVs (80, 87, 136, 145, 146, 213, 493, 542, 692), all of them agreed that both kits are useful to investigate outbreaks of gastroenteritis, but not in case of sporadic cases (Table 1.2).

### 1.1.5.1.3. Monoclonal antibodies

Use of mAbs against HuNoV and HuSaV strains has provided answers to different questions in virus-cell interaction and antigen diversity, but they have been mainly developed to increase virus detection sensitivity. Treanor et al (1988) and Herrmann et al (1995) produced mAbs against SMV and NV strain, respectively, and demonstrated that cross reactivity in both directions did not occur (267, 642). Antigenic mapping of rNV (NoV GI) and recombinant Mexico (rMX, NoV GII) capsid protein were performed with mAbs. Ten mAbs to rNV were characterized by ELISA, Western Blot (WB) and immunoprecipitation, differentiating between continuous and
discontinuous epitopes. Seven of them recognized discontinuous epitopes, whereas the other three recognized continuous epitopes (260). Eight of them mapped to the C-terminal region of VP1, suggesting that immunodominant epitopes were located in that region. Similar for rMX, 10 mAbs recognizing continuous epitopes were characterized. However these mAbs mapped to the N-terminal region of VP1, suggesting that antigenic epitopes were located here instead of the C-terminal region (714). Moreover, 2 mAbs (1B4 and 1F6) were able to detect other NoV GII strains as well as NoV GI, suggesting a possible common epitope between these two genogroups. As a follow up, the same research group characterized 17 mAbs that recognized 16 continuous and 1 discontinuous epitope on NV VP1 (713) and demonstrated that 2 of these mAbs recognized a panel of different NoV GI and GII strains, being useful to detect a broad range of NoVs (711).

Another cross reactivity study among several strains was conducted by Kitamoto et al (2002) (354). Strain- genogroup- and genus (NoV or SaV) specific mAbs were produced by oral inoculation of mice with either a single rNV, rKashiwa, rSMV or rSV-VLPs or a mixture of two different genogroups. The mAbs were classified as those that recognized GI and GII strains (group A), those that recognized GI (group B) or GII (group C), and those that recognized only one strain (group D and E). Particularly, those mAbs from mice inoculated with rSV, reacted with rSV, but none of the recombinant NoVs.

The mAb technology also has been useful to recognize binding epitopes on VP1. Two mAbs, one against NoV GI-1 and another against NoV GII.2 were mapped to the P2 domain of VP1 and blocked binding of rVLPs to Caco-2 cells. Moreover the mAb 61.21 (against NoV GII.2) recognized an epitope completely conserved among NoVs strains across genogroups (412). Other common epitopes between NoV GI and GII or GI and
GIII have been described (33, 496, 507). The description of a common genogroup epitope and genogroup specific epitopes opens the possibility for development of ELISA kits for rapid screening of several samples. This is partially achieved by the current commercially available kits (22).

1.1.5.1.4. Hemagglutination inhibition

Norwalk virus VLPs bind and hemagglutinate red blood cells (RBCs) of humans and chimpanzees, but not other species, (295). Binding is mediated by recognition of carbohydrates present in the membrane of RBCs as part of the HBGAs (629). Norwalk VLPs agglutinated 100% of human RBCs with type O, A and AB, but only 29% (4/14) of type B. Also H type 2 antibodies blocked the binding and agglutination of Type O RBCs. Overall these were the first reports about the role of HBGAs, also present in the gut, in NoV binding. Further studies demonstrated that susceptibility to infection by NoVs depends on the host HBGAs and the NoV strain (section 1.1.7.2).

1.1.5.2. Nucleic acid detection: molecular techniques

1.1.5.2.1. Natural RTPCR inhibitors: problem and possible solutions

From the well known RTPCR to a recently developed rt-RTPCR, these molecular techniques are powerful methods to detect microorganisms in biological materials such as clinical, food and environmental samples. However the major disadvantage to direct RTPCR amplification of viruses in clinical (fecal) samples is the presence of PCR or RTPCR inhibitors. More complex is the case of food and environmental samples where a low virus concentration is an additional problem besides the presence of PCR inhibitors (85).
The RT-PCR inhibitors include bile salts and complex polysaccharides in feces (376, 459), humic and fulvic acids in soil (646, 678), proteinases in milk and oysters (344) and urea in urine (349) among many others (695). When present in the sample, inhibitors prevent amplification of the target sequence by: (i) interfering with cell lysis; (ii) capturing or degradation of nucleic acid target; (iii) inhibition of polymerase activity or a combination of these (695).

Many techniques to remove inhibitors have been developed (695), and although each method is unique in one or other steps, all of them will first dilute, separate or elute the pathogen from the rest of the sample and PCR inhibitors. This will not be a problem for further detection in the case of fecal samples, since the pathogen, AECV or HECV, is present in higher concentrations (3x10^8 copies/gram) (99). However in food and environmental samples, it is necessary to include a concentration step to recover the nucleic acids, because the level of contamination is low. For feces the most common technique includes sample dilution in phosphate-buffered saline (PBS) or minimum essential medium (MEM), and disruption of viral the particle by TRIZOL ® LS (Invitrogen Inc), chloroform/isopropanol extraction and recovery of RNA in aqueous solution. After that RNA purification may be achieved by linking of RNA to a silica gel membrane. However many other techniques have been developed (Table 1.3). In the case of food and environmental samples, the first step is achieved by direct elution, absorption-elution or centrifugation (553, 556, 587). The follow concentration steps include precipitation by polyethylene glycol (PEG) (352) or paramagnetic beads (358), organic flocculation (23) or ultracentrifugation (386). After that, extraction and
puriﬁcation of nucleic acids is performed by routine methods or commercially available
kits (24, 386) (Table 1.3).

However no universal methods have been developed and none of the available
methods can insure eﬃcient removal of natural inhibitors. Several strategies have been
developed to assess the presence of RT-PCR inhibitors including: (i) addition of a target
RNA before ampliﬁcation (185); (ii) use of diﬀerent primer pairs to amplify a second
target likely to be present in the sample (402); and (iii) addition of an internal control
(InC) (277). Among these approaches, the use of an InC that is co-ampliﬁed with the
target RNA has been successfully used to detect the presence of inhibitors in clinical
samples (106, 162, 674) and in environmental samples (509, 575, 576) when assessing
the presence of HECV or AECV.

1.1.5.2.2. RTPCR

Today RTPCR is the most widely used assay to detect ECV in clinical, food and
environmental samples (21, 48, 58, 155, 195, 208, 255, 317, 346, 366, 379, 391, 392,
413, 439). For diagnosis purpose, virus-speciﬁc primers are mainly used to amplify
conserved regions within the RdRp. However when the goal of the RTPCR is virus
classiﬁcation, primers target the ORF2 of NoVs or the 3’end of ORF1 of SaVs that
encodes for the VP1 (capsid). The genome diversity among NoVs and SaVs has made it
impossible to design a single pair of primers that can detect the whole spectrum of
viruses (274, 668) and many primers have been developed and published.

The sensitivity and speciﬁcity of a given RTPCR protocol are keys for detection,
but they are especially important in the case of ECV, given their genetic variability and
low concentrations particularly in non clinical samples. The term sensitivity refers to the ability of the RTPCR to detect both weak and strong positive samples, as well as the ability to detect a broad range of genotypes (145). This can be achieved by testing a given RTPCR protocol against a panel of NoVs and SaVs of known genotype or using a large number of samples collected from different scenarios (outbreaks, sporadic cases, age, food, water, etc) and compare the results with a gold standard technique (usually EM) (145). Although the second criteria is usually chosen, because of the difficulty of access to a panel of different known genotypes, several studies have compared either or both aspects of sensitivity of different RTPCR protocols. The performance of five different RTPCR protocols, that included 18 primers arranged into 11 different pairs was assayed using a panel of 82 stool samples collected over a 4-year period of time from outbreaks and sporadic cases in Europe and previously tested positive by EM and/or RTPCR (668). Overall, considering the five protocols together, the RTPCR sensitivity was 84% (69/82), but the individual RTPCR sensitivity ranged from 52% to 73%. When the sensitivity was examined for each genogroup, single RTPCR sensitivity ranged from 54% to 100% for GI and 58% to 85% for GII. Using a panel of 116 NoV or SaV positive stool specimens (11 outbreaks and 60 sporadic cases in Japan), Honma et al evaluated 9 different sets of primers targeting the RdRp. No single primer pair detected all the positive samples and at least 3 primer pairs were required to detect NoVs and SaVs (274).

The results observed in another study in Germany differed after testing 244 watery stool samples from 2 gastroenteritis outbreaks by EM, ELISA and RTPCR. In this case RTPCR had the highest sensitivity (94.1%) followed by EM (58.3%) and ELISA (31.3%) (534). This was not surprising because all the samples belong to the same
outbreaks, reducing strain variability and increasing the RTPCR ability to detect virus. In another study by Gallimore et al, 22/71 EM-positive samples were negative by ELISA. When the authors assayed those 22 samples by RT-PCR, 10/22 (45%) were characterized as GRV strain (NoV GII), but 12/22 (55%) were HuNoVs different from the GRV strain (195). Overall these results indicate that although RTPCR is more sensitive than classical techniques, no single pair of primers detected all the circulating strains, and negative samples from or related to outbreaks matching Kaplan’s criteria (337) should always be retested with additional primers (381, 594).

Broadly reactive and degenerate primers that can detect NoVs and SaVs have also been described. A set of primers, p289/p290, were designed by Jiang et al (317) to amplified HuNoVs GI and GII and also HuSaVs. Latter these primers were used to detect AECV, although with less efficiency than those specifically designed for BoNoVs (594), PoNoVs and PoSaVs (677). Le Guyader et al (379) evaluated a degenerate reverse primer, p110, that can prime cDNA synthesis from HuNoVs GI, GII and HuSaVs and it has been widely used in food and environmental samples (371, 383, 413). Another primer pair, JV12/JV13 (666) and its modification JV12Y/JV13I (660) are able to detect NoVs GI and GII and have been applied to clinical, food and environmental samples (61, 64, 446). However, because these broadly reactive and degenerate primers have mismatches with the target sequence, RTPCR is performed under low stringency, increasing the chance to detect the target, but also the chance of detecting non-specific amplicons. For example, primer set p289/p290 could also detect RV (423), and primer p110/Sapp36 will cross-react with enterovirus (EV) (274). Because of this, it is necessary to confirm the RTPCR results by other techniques such as heteroduplex mobility assay (HMA), dot-blot
hybridization, microwell probe capture hybridization or sequencing (see section 1.1.5.2.4 and 1.1.5.2.5) (381, 667).

In general RT-PCR can detect $10^{1-3}$ particles/g or ml of sample. Few studies have described the quantity of the virus genome that must be present in order to detect the virus. Le Guyader et al reported 10- to 1,000-fold differences in the quantity of different NoVs strains that could be detected by two primer sets. The authors observed that when large quantities of virus were present both primer sets could detect it, but when small quantities were present, only primers with high homology were useful (379). To improve sensitivity (defined as the ability to detect low amounts of target) particularly in the case of non clinical samples, several techniques have been developed. Nested-RTPCR has been shown to be 10-1000 times more sensitive than RTPCR for detection of NoVs in seeding experiments with shellfish (227). Another nested-RTPCR was developed by Medici et al (446), using JV12/JV13 as RTPCR primers and 9 different primers (5 forward and 4 reverse) for the second round. Sensitivity was comparable to RTPCR followed by SB and at least 10 times higher than RTPCR. Heminested multiplex RTPCR to simultaneously detect and differentiate NoVs GI and GII has been able to detect 20 viral particles/ul in stool samples and potentially in environmental samples (485, 707, 717). Booster-RTPCR and booster-nested-RTPCR, consisting of a second round-PCR or nested-PCR, respectively, were also developed for NoV and SaVs (138, 276).

Among the newest applied techniques for detection of NoVs are transcriptional enhancement of RTPCR (TE/RTPCR) (311), reverse transcription Loop-mediated Isothermal amplification (RT-LAMP) (712) and nucleic acid sequence-base amplification (NASBA) (312, 369, 460, 554). Although recently developed, the three technologies are
potentially useful to test food samples, because of their high sensitivity. Particularly the NASBA system in a multiplex format has been demonstrated to detect NoVs GI and GII simultaneously at initial inoculum levels of $10^0$ to $10^2$ RTPCR-detectable units, with the advantage that the reaction is performed at a single temperature and it can achieve an amplification of $10^9$ fold in 1.5-2h (123, 312).

### 1.1.5.2.3. Real-time

Real-time-RTPCR (rt-RTPCR) has been developed to detect HuNoVs from fecal samples and shellfish (243, 244, 273, 414, 452, 502). The rt-RTPCR was reported to be 10,000 times more sensitive than RT-PCR (502), faster, quantitative and it did not require a confirmation step. Both SYBR green and Taq Man rt-RTPCR have been developed, as well as a multiplex rt-RTPCR (45, 504). Several studies have compared the sensitivity (as the ability to detect weak positive samples) of SYBR green and Taq Man rt-RTPCR with conventional RTPCR. The SYBR green rt-RTPCR has lower, same or higher sensitivity than conventional RTPCR depending on the study. Gunson et al (243) found that SYBR green rt-RTPCR was less sensitive than conventional RTPCR with primers JV12Y/JV13I described by Vennema et al (660) when applied to fecal samples positive for NoVs by EM. Smith et al (569) found his rt-RTPCR as sensitive as a conventional nested-RTPCR, whereas Beuret et al (45) indicated that multiplex rt-RTPCR was more sensitive than conventional RTPCR for NoVs GII. Almost the same results were found when Taq Man rt-RTPCR was compared to conventional RTPCR and nested-RTPCR in shellfish and faecal samples (325, 414, 504). The ability to detect a broad range of genotypes was also studied,
resulting in 3-5 GI and 8-10 GII genotypes (depending on the study) detected by SYBR green, and 4GI and 7GII genotypes detected by Taq Man rt-RTPCR (273, 328, 504, 543).

**1.1.5.2.4. Hybridization assays (solid, liquid and microwell capture format)**

Hybridization assays were developed mainly with two objectives: to increase RTPCR detection level and to confirm RTPCR products. However the different formats developed over the time have allowed researchers to also apply hybridization for screening of large numbers of samples, classification and detection of more than one strain present in one sample.

Application of RTPCR followed by Southern blot (SB) hybridization to fecal samples was first described by Ando et al (1995). Based on the sequence of 20 NoV strains, four probes [P1-A: GI-1/4/7; P1-B: GII-6; P2-A: GII-3 (Toronto-like); P2-B: GII-1/2/3(Arg-320-like)/4/5/8/10/11/12/14/15] were designed for SB and detection by chemiluminescence. Correlation with prior SPIEM results demonstrated that the antigenic type of the NoVs could be predicted by this technique (14). The technique was subsequently applied to trace back a shellfish-gastroenteritis outbreak in the U.S. and to assess viral contamination in shellfish beds during different seasons along the French coast (13, 380). Other authors have demonstrated season variability by using RTPCR followed by SB. In Japan, 47 outbreaks of NoVs gastroenteritis were caused by P2-B strains in 1996-1997, P2-A between 1997-1998 and P1-B between 1998-1999, and P2-B since 2000 until 2003 (299, 300). A similar approach, reverse line blot hybridization (RLB) using 5’ biotin-labeled RTPCR products instead of labeled probes was proposed
by Vinje et al 2000 (667). Briefly, each 5’labeled RTPCR product was hybridized to a panel of 18 probes (genogroup and genotype specifics) bound to a nylon membrane and after hybridization detected by chemiluminescence assay. The technique showed the same sensitivity as classical SB, but with three advantages: less time consuming, because the membranes can be prepared and stored in advance and only the RTPCR product has to be added; cheaper, because membranes can be reused, and more flexible, because an emerging genogroup or genotype probe can be added to the existing format.

The microwell capture hybridization format added more simplicity and accuracy to the classical hybridization, because the visual interpretation of a band in a gel is replaced by a numerical value based on absorbance intensity and a defined cut off. Different formats have been developed, but all of them have in common the detection of a pre amplified RTPCR product by a labeled antibody using an ELISA format. Maunula et al 1999 (439), used 5’biotin-labeled forward primers to obtain biotinylated RTPCR amplicons that were applied to streptavidin-coated plates. Then 7 digoxigenin-labeled genogroup and genotypes specific probes were individually added and positive hybridization was developed by luminescence. Latter the same format was used with 10 probes [including P1-A, P1-B, P2-A, P2-B described by Ando et al 1995 (14) and by Fukuda et al 2002 (188). Both authors indicated that microwell capture hybridization was more sensitive than RTPCR, but similar to SB. However, they also indicated that no cross-hybridization was detected and the results were achieved in less than 12hs, compared with 1-2 days required for SB. A variation of this format was introduced by Schwab et al 2001 (578) and Wang et al 2006 (674), where the different probes are bound to the plate directly or by a streptavidin-biotin interaction. In the first case the 5’biotin-
labeled RTPCR amplicons, or in the second case, the non-labeled RT-PCR amplicons are added and the hybridization reaction was developed by a Neutravidin®-Horseradish peroxidase (HRP) conjugate or an anti-dsDNA antibody and protein A-HRP conjugate, respectively. Again sensitivity was higher than RTPCR but not higher than SB. However both methodologies are less time consuming because plates can be prepared and stored for a long time and several samples can be tested simultaneously for several strains. The microwell hybridization developed by Schwab et al detected NoVs and HAV in stool and environmental samples, whereas the technique described by Wang et al is the only one to apply this format to detect PoNoVs and PoSaV in feces.

A recently developed method combines liquid hybridization with electrochemiluminescence (ECL), a new technology developed by BioMerieux (311). In this case, a 3’end of the forward primer used during RTPCR is linked to a specific sequence that will be recognized during the hybridization by a detector probe. The hybridization using the ECL principle applies two probes: (i) a specific 5’ biotinylated capture probe immobilized onto streptavidin-coated paramagnetic beads; and (ii) a detector probe complexed to a ruthenium chelate (Ru$^{2+}$). When incubated with the RTPCR amplicon, the biotinylated probe will hybridize both the amplicon and the streptavidin-magnetic beads, and the detector probe-Ru$^{2+}$ will hybridize the 3’end of the amplicon. The paramagnetic beads carrying the hybridized amplicon/ECL probe complex are magnetically captured in the surface of an electrode. Voltage applied to the electrode triggers the ECL reaction that results in light emitted by the Ru$^{2+}$-labeled probes proportional to the quantity of amplicon present. Detection limits improved by 1 log$_{10}$ and the ECL appears to be a promising method for environmental samples where virus concentrations are very low (236, 311).
1.1.5.2.5. Heteroduplex motility assay

Heteroduplex mobility assay is a rapid and cheaper alternative to sequencing analysis. Basically the migration of a discrete double-stranded DNA molecule, through a non-denaturing electrophoretic gel is influenced by the degree of base-pairing of the double helix. Amplicons that have exact complementary single strands, between a known and unknown strain, will migrate more quickly than identically sized amplicons that have mismatched bases. The limitation of this technique resides in a need for known sequences. Although it is not a definitive classification, the technique is useful for first screening of a large number of samples and to identify samples with multiple strains (685). It was applied to characterize NoV RTPCR products from 130 outbreaks in England between 1997-1999. The authors classified the samples into 3 groups: GRV-like strains (68/130), MxV-like strains (3/130) and non GRV-non MxV-like strains (49/130) (434). Green et al (2004), demonstrated the utility of this technique to assess the simultaneous presence of several NoV strains in sewage and sea water, where usually more than one strain is present (226). The technique was also used to identify new strains in 529 outbreaks and 141 sporadic cases of NoV gastroenteritis in the North of England between 1998-2001 and 223 outbreaks in the North-West of England between January, 2000 and July, 2001 (194, 195).

1.1.5.3. Future opportunities: microarrays

Microarrays have already been used for the detection and characterization of rotaviruses, influenza, zoonotic and respiratory viruses (114, 581, 671, 696). For NoVs a first attempt to apply microarrays for detection resulted in an overall fast and specific
protocol (305). In this protocol, RTPCR amplicons were synthesized with 3’ end modified reverse primers that contain T3 RNA polymerase promoter sequence. These RTPCR amplicons are transcribed into ssRNA and hybridized to the array where genogroup and genotype specific primers are spotted. Reverse transcriptase will add fluorescent nucleotides to the overhanging T3 polymerase promoter sequence, and after washing, fluorescence from hybridized products is detected using a microarray scanner. The system was able to accurately genotype 19/19 previously genotyped samples. In a batch of 74 samples submitted for analysis during 1998-2004, 45 were positive by microarray versus only 35 by conventional RTPCR. However in 109 samples positive for NoVs by EM collected between 1977-1997 only 36 were positive by microarray. Overall genogroup specificity was good, no cross-reactions were observed and double infections were detected. However, additional efforts are needed to improve sensitivity and deal with sequence issues in primer design because if hybridization is incomplete, primer extension and fluorescence nucleotide addition will not occur (511).

In the food and environmental sciences, although microarrays will be very useful, several challenges such as the complexity of the food matrix, the low amounts and genetic variability of the pathogen recovered, and the cost of a microarray assay itself, will have to be considered before it can be applied (368).

1.1.6. EPIDEMIOLOGY

1.1.6.1. Incidence and prevalence

The number of cases of gastroenteritis in Australia have been estimated as 17.2 million, whereas in U.S. the total were 38.9 million (445, 500). Human NoVs are
recognized as the major cause of epidemic non bacterial gastroenteritis worldwide being responsible for 73% to 95% of the outbreaks, and half of all gastroenteritis outbreaks (54, 172, 173, 421). They are also recognized as the major cause of sporadic cases of gastroenteritis, and the most common viral cause of gastroenteritis identified in community-based studies (22). Most of the infections and outbreaks are caused by a single strain, although co-infections occur, and although many strains co-circulate, GII.4 NoVs have emerged, evolved and remain as the principal genotype that causes outbreaks worldwide (418, 686). The greatest morbidity and economic impact of NoV gastroenteritis are among the elderly in nursing homes, leading to an increased need for hospital care and increased mortality (422, 702). In addition nosocomial outbreaks in hospitals and care centers have also increased, accounting for approximately 40% of NoV outbreaks in the U.S. (172), England (420) and the rest of Europe (419). Although outbreaks can occur year around, there is a marked increase in the number of outbreaks during winter and early spring with a peak between January and March. Also the number of people involved in one outbreak vary from 10 to thousands (173). The *Norovirus* genus is considered the single virus genus responsible for 50-66% of foodborne illness in the U.S. (688).

Sapoviruses are also associated with gastroenteritis (112). Although with lower incidence and prevalence, HuSaVs have been associated with gastroenteritis outbreaks (0.01-9%) and sporadic cases (0.4- 6.6%) (54, 256). Outbreaks have occurred in day-care centers, healthcare facilities and schools, but given the low prevalence and incidence, no information is available about the impact on each setting (7, 196, 197, 257, 323, 458). Fewer foodborne outbreaks are associated with SaVs.
In the veterinary field, PoNoVs were detected in Japan and Europe and recently in the U.S. (621, 677). Only one study has assessed the presence of PoNoVs and they were detected exclusively in fecal samples from finisher pigs with an overall prevalence of 20% (range, 0-40% among farms and 3-40% among NoV-positive farms), although samples from other ages of pigs were also collected at the same time from the same farms (677). Most positive samples in this study were from healthy animals suggesting that, as previously observed for HuNoV infections (546), asymptomatic shedding of PoNoV occurs in adults contributing to virus persistence in the field. Different is the case of PoSaV that has emerged as an important pathogen associated with diarrhea and subclinical infections among pigs of all ages (256, 433, 677). The PoSaV/Cowden strain (246), was first reported by Saif and colleagues (1980), based on its calicivirus morphology by EM of diarrheic stools of pigs in the U.S. In a small-scale survey of SaVs in three Ohio swine farms (Guo and Saif, unpublished), PoSaV was detected in 51% of 156 fecal samples mainly from nursing pigs (48%) and post-weaning pigs (75%), but not from adult pigs in one farm. The PoSaV was detected from 100% of diarrheic nursing pigs and 50% of normal nursing pigs, and from 89% of diarrheic post-weaning pigs and 69% of normal post-weaning pigs in the same farm. In this farm, 80-100% of pigs in all age groups were seropositive for PoSaV antibodies indicative of high exposure rates. These limited findings suggest that PoSaV may be a major cause of post-weaning and nursing pig diarrhea, but subclinical PoSaV infections also occur. In a second survey, 68% of fecal samples collected from healthy pigs were positive for PoSaV (677). But in contrast to PoNoV, PoSaV was detected in feces of all age groups: 28% in nursing; 86% in post-weaning; 74% in finisher pigs and 67% in sows. Porcine SaV have also been
detected in Venezuela and Korea, where prevalence was lower than in U.S. (18% and 4.9%, respectively), probably because of primer sensitivity (350, 433). Seroprevalence studies for PoNoV have detected antibodies against SW918, (a PoNoV GII strain detected in Japan), in 83% of sera from pigs in the U.S. and antibodies against Hawaii strain (HuNoV GII) in 52% of these samples. Surprisingly, 63% of sera also reacted with NV virus (HuNoV GI), a genogroup that have not been detected in swine and suggest the possibility of interspecies transmission (174).

There is little information about BoNoVs, besides the original reports that indicated the detection of BoNoVs in stool samples of diarrheic calves (297, 594, 652) and a seroprevalence study in Germany that showed an unexpectedly high prevalence (99.1%) of antibodies against Bo/NoV/Jena 117/80/GE, suggesting a constant exposure to the virus (142). The prevalence of BoNoVs was high among young veal calves (72%) in the U.S. (594), similar to the findings reported by van der Poel et al in Germany, where the prevalence was 44% (653). More recently studies in South Korea (506) and Germany (142) showed a lower prevalence for BoNoVs (9.3% and 8.9%, respectively). Interesting is the fact that strains detected in the U.S. (594) as well as 90% of those detected in South Korea (506) belong to NoVs GI.2, whereas those detected in Germany belong to NoV GI.1 (142).

### 1.1.6.2. Transmission and sources

Person-to-person, airborne transmission, contaminated food and water and fecal-oral routes are the more common routes of transmission among other unknown routes (54, 172, 173). Although the role of each route has changed over the years, the primary
route of transmission of NoVs is the fecal-oral route. Usually NoV is present in the stool of infected, [but many times asymptomatic people, (666)] and vomitus. Primary infection results from the ingestion of contaminated food or water (12, 270). Secondary infection results via person-to-person contact, aerosol, fomites and infected food handlers (35, 131, 170, 269).

Food-borne transmission can be divided into two categories: (i) pre-harvest, when the contamination of the food occurs before they are harvested, such as the case of shellfish growing in contaminated waters, or soft fruits irrigated or sprayed with contaminated water; or (ii) harvest or processing, at any point from the moment of the harvest until the final product, including harvesters, plant workers, food handlers, chefs, etc. (41, 93, 202). Waterborne outbreaks also can be divided in those that occur by: (i) drinking of contaminated water, or (ii) by contact with contaminated recreational water (272, 438).

Environmental surfaces also play a role in outbreaks at health care facilities (198), recreational and vacation places, cruise ship because of the high stability of NoVs on surfaces (557). Sometimes, combinations of many of these routes result in major outbreaks, very difficult to control, such as the case of the cruise ships. In these cases, the combination of person-to person transmission, environmental contamination and aerosol transmission results in multiple, prolonged and severe epidemics of acute gastroenteritis (AGE) (302). Transmission is enhanced by several factors such as the low infectious dose, asymptomatic shedding, environmental survival and strain diversity (section 1.1.7.1) (31, 231, 455, 513, 720).
1.1.6.3. Surveillance

Surveillance of infectious disease can be defined as the ongoing and systematic collection and analysis of data, and dissemination of the results to avoid or prevent infections or epidemics (469). Although both NoVs and SaVs cause gastroenteritis in humans, most if not all surveillance for gastroenteritis are focused on NoVs given their high impact. Both active and passive surveillance systems have been applied to determine the emerging trends in gastroenteritis, and particularly foodborne disease (443).

In U.S. the Foodborne Disease Active Surveillance Network was established in 1996 to determine the burden of gastroenteritis and foodborne disease. Between 250 and 350 million people suffer AGE annually in the U.S., and an estimated 22% to 20% of these cases are thought to be foodborne. This results in ~76 million foodborne illnesses, more than 300,000 hospitalizations and 5,000 deaths per year (445). The total annual estimated cost of foodborne outbreaks is between $2 and $4 billion (82). Despite many efforts 82%, of foodborne illnesses are of unknown etiology (445). Between 1996 and 2004, a subset of 549 out of 674 outbreaks of NoV AGE was studied. Outbreaks were reported from 33 states between January 1996 and June 1997 (173), 29 states, the District of Columbia, the US Virgin Islands and cruise ships docking at American ports between July 1997 and June 2000 (172), and 33 states, the District of Columbia, Puerto Rico and cruise ships between July 2000 and June 2004 (54). The most frequent settings were nursing homes, hospitals and restaurants, among others (Figure 1.7). Persons of all ages (6 months to 101 years-old) were affected and the median number of people affected per outbreak was 50 (range 10-6000) between 1996 and 1997, 40 (range 4-800) between 1997 and 2000 and 45 (range 4-547) between 2000 and 2004. Contaminated food and
person-to-person contact were the most common forms of transmission, although differences were observed in each period of time and no mode of transmission could be determined in 24-30% of the outbreaks (Figure 1.8). Outbreaks occurred throughout the year, with a winter/spring seasonality between 1996 and 1997 and 2000-2004, but no distinct seasonality between 1997 and 2000 (54, 172, 173).

Similar studies in Europe involving 10 countries where the number of AGE illness varies from 9.4 million cases/year in England (2.1 million foodborne cases), 4.5 millions cases/year in The Netherlands and 3.2 million cases/year in Ireland showed that NoVs are responsible for >85% of non bacterial AGE (421). The percentage of outbreaks associated with food- or water-borne transmission varies with the country. Finland, England, Slovenia, Spain and The Netherlands reported 7% to 24% of AGE to be related to food and water, whereas the percentage increases to 94%-100% in France and Denmark. This is in part because surveillance systems in the latter two countries are designed to detect foodborne disease, whereas in the other countries the data is general about outbreaks, independently of the mode of transmission. The distribution of settings is also a reflection of this. Whereas in Denmark 75% of the outbreaks occur in food outlets, outbreaks in England, The Netherlands and Spain are more frequent in residential homes and hospitals (73, 421, 624). Outbreaks occurred throughout the year, and a winter seasonality was observed in the case of Germany, The Netherlands and England (296, 420, 624).

The estimated incidence of AGE in Australia is 17.2 million cases/per year, whereas foodborne illness accounts for 5.4 million cases, 15,000 hospitalizations and 80 deaths annually. Noroviruses account for an average of 1,832,000 cases of AGE, which is
200,000 cases more than the number of cases associated with bacterial AGE, including *Campylobacter spp, Salmonella spp, STEC* and *non-STEC Escherichia coli, Shigella spp, Staphylococcus aureus, Vibrio parahaemolyticus*, etc. (253). Of the 5.4 million foodborne cases, a known pathogen is recognized only in 28% of the cases (1,480,000 cases) and NoVs account for 30% of them after *non-STEC E coli*.

Many other studies have assessed the presence of NoVs from different and potential (in many cases late confirmed) sources of infection. In some cases the studies have demonstrated seasonal (46, 586) and/or geographical differences (59, 60, 111, 184, 380), and in others they have also linked an outbreak to a distant geographical point (64, 649). In others, the authors have suggested the possible source of multiple strains and the risk of recombination (329, 476, 477, 625).

### 1.1.6.4. Outbreak investigation

#### 1.1.6.4.1. Foodborne outbreaks

The NoVs and HAV are the most common causes of foodborne viral gastroenteritis and hepatitis, respectively, worldwide (363, 688). Despite major advances and improvements in food and water quality (363), diagnostic methods (553, 622) and surveillance systems (92, 443), foodborne diseases remain a global public health problem (54, 363). Changes in the scale of production, processing and global distribution have enabled the food industry to supply consumers with fresh products in most countries year-round. However globalization has also increased the risk for human illness associated with food. Three outbreaks associated with a national sandwich restaurant franchise in Michigan (93), a multistate outbreak due to contaminated oysters harvested
in Louisiana (94), and six outbreaks of gastroenteritis in Denmark due to frozen raspberries imported from Poland (171, 367) are examples of local, national and international outbreaks associated with consumption of contaminated food.

Three major routes for food contamination have been described: (i) shellfish contaminated by fecally polluted marine waters; (ii) sewage pollution of irrigation waters; and (iii) ready to eat (RTE) and prepared food contaminated during food preparation. (564). The potential for foodborne transmission of NoVs was first suspected after a AGE outbreak in the U.K. in 1976, where the consumption of sewage-contaminated oysters was the suspected cause (18). The first recognized AGE outbreak due to consumption of contaminated food was in Australia affecting at least 2000 people between June and July 1978 (464). The source of virus implicated in this outbreak was seafood, particularly oysters and other shellfish. In the U.S. the first outbreak with these characteristics occurred in 1980 (242). Bivalve molluscan shellfish are filter feeders capable of concentrating particles present in the surrounding waters by more than four-fold, including fecal coliforms and viruses (77), acting as passive vehicles for enteric disease transmission. The percent of the outbreaks associated with seafood is around 10-20% in countries such as the U.S. or Australia, but this percentage increases to 70% in countries with higher seafood consumption such as Japan or wherever seafood is eaten raw (81, 389). Outbreaks associated with consumption of shellfish are continuously reported worldwide (16, 41, 88, 90, 91, 98, 111, 153, 193, 206, 207, 264, 361, 383, 384, 463, 471, 498, 530, 590, 612, 625, 679). Despite differences in the number of people affected, seasonality (not always observed) and the source of shellfish contamination, all the NoV-shellfish associated outbreaks show 3 characteristics: (i) the implicated shellfish
were often consumed raw or partially cooked (353, 444); (ii) people in several areas could be affected because of the distribution of products (41, 679); and (iii) depuration was not effective to remove the virus (237, 382).

Besides shellfish, outbreaks are also associated with contaminated sandwiches (199, 238, 505), fruits (127, 171, 270, 367, 385), vegetables (8, 591), bakery products (11, 373), and cold meats (577). One of the largest outbreaks related to contaminated vegetables was reported from Japan, where 660 people who ate lunch prepared by a restaurant during a 2-day period developed illness (269). In another outbreak, 125 university students became infected after eating contaminated ham served in the delicatessen sandwiches (131). The contamination of RTE food, particularly fruit and vegetables, comes from products grown in fields irrigated with wastewater, but the most common source of contamination comes from poor hand-washing practices of food-handlers. Most of these workers were ill previously or at the time of preparation of the food. As an example 48 people became ill after a Christmas party. The epidemiological investigation showed that the salad was strongly associated with the disease and the only food handler that prepared salad became ill 30 min after finishing his shift, suggesting that virus shedding occurred before clinical signs (201). Moreover the prolonged asymptomatic shedding detected in patients after a clinical AGE, significantly increase the opportunity of an asymptomatic food handler to continue to contaminate food during preparation (304).
1.1.6.4.2. Waterborne outbreaks

Viruses entering the sewerage system may survive wastewater treatments and contaminate receiving waters. The NoVs have been detected in sewage influent and effluent on the order of $10^7$ virus units by PCR. (413) and $1$ log lower in the treated effluent. Therefore, they will represent a problem to recreational users, shellfish consumers, etc. Later, NoVs can also survive further treatments of drinking water (usually by filtration and chlorination). Actually NoVs were found to be relatively insensitive to chlorination treatment (348). Nevertheless, in most cases contamination of “clean” water occurs after treatment because: (i) heavy rainfall or floods overwhelm treatment plants (370, 371); (ii) failures in treatment process (338); (iii) exceptionally high levels of virus (517); and (iv) virus leakage from sewers or septic tanks (266).

Between 1980 and 1994, 28 outbreaks affecting 11,195 people were recognized in the U.S., with 9,000 of these attributed to NoVs. Sewage contaminated ground water was the source of a NoV outbreak affecting 1,450 people in Ohio (486). Another water-borne outbreak was linked to contaminated well water, attributed to the geological formations of the area and the overloaded sewage disposal system in Wyoming. The outbreak continued over 10 weeks and the epidemic curve was consistent with continued exposure, that also explains the detection of several NoV strains (12). Other outbreaks due to contaminated water have been reported in Sweden (483), Switzerland (251), etc. Outbreaks in swimming pools and recreational waters with secondary person-to person spread have been reported in Europe and the U.S. (271, 437, 573, 715) as well as many outbreaks associated with drinking contaminated drinking water (47, 55, 370, 371, 442, 574).
1.1.6.4.3. Other outbreaks

Many other neither food- nor water-borne outbreaks have been described. Transmission of NoVs during a football game from the visiting team to the home team have been documented (35). Studies of an AGE affecting half of the patients and the staff of a hospital ward for the mentally infirm demonstrated the presence of NoVs in samples collected from the ward environment, including lockers, curtains and commodes (229). Evidence for airborne transmission has been collected. Three hundred people developed AGE from one person who vomited during a performance in a concert hall (170); a restaurant outbreak was also related to a customer vomiting during dinner (429) and a 20-days long school outbreak, involving 492 students in 15 classrooms divided between 2 buildings was also the consequence of vomiting (430). Person-to person transmission is the most probable route in institutional settings like hospitals and nursing homes or places where many people gather together (395, 436, 549). Rapid propagation of NoV AGE illness through 29 nursing homes following a pilgrimage to France was described in Switzerland. The number of cases reached 69 at the end of the 4 day trip; during the following 10 days the disease spread through the nursing homes reaching 380 apparently secondary cases. Among the first 69 cases, 33 people shared the third floor of the hospital where they were accommodated when they arrived, and the other 36 were accommodated in nearby hotels. No cases occurred among pilgrims in other floors or among other hotels (186). A large outbreak involving more than 1000 people during a period of 10 days was reported among evacuees of Hurricane Katrina who were sheltered in the Reliant Park Complex in Houston, TX (709). On Sept 2, two days after evacuees arrived, an increasing number of clinic visits for AGE was noticed, and surveillance as well as different
intervention measures to reduce the spread of the disease were introduced. The outbreak reached a peak on Sept 5, and despite major efforts, the outbreak continued until the clinic was closed on Sept 12. The epidemiologic characteristics of this outbreak suggested person-to-person, contact with contaminated surfaces and airborne transmission as the main routes of spread, and also the detection of different strains during the 10 days outbreak, indicated multiple introductions of the virus.

Another excellent setting for NoV AGE has been and continuous to be cruise ships. The U.S. Centers for Disease Control and Prevention (CDC) in Atlanta, Georgia, has operated the Vessel Sanitation Program since 1973, which resulted in a steady decline in NoV outbreaks until 2000 (96). Between 2001 and 2004, however, the number increased almost 10-fold, according to CDC researchers. Around 2002, noroviruses also began striking with increasing frequency on European cruise ships (364, 686). In 2002, between January 1 and December 2, 14 outbreaks of AGE that occurred on cruise ships were analyzed. Noroviruses or SaVs accounted for 12/14 outbreaks. Six of twelve outbreaks compromised more than 2 contiguous cruises on the same ship with 100% new passengers on each cruise, and 1 outbreak continued for 42 days in 4 contiguous cruises of the same ship (686). In 83% of the outbreaks, person-to-person transmission was suspected, whereas environmental contamination was suspected in 50% of the outbreaks and food- and water- borne transmission in 2% and 3%, respectively, suggesting that more than one route was involved. Another outbreak affected passengers in 2 consecutive cruises of a ship and continued on 4 more cruises despite 1 week of aggressive sanitation (302). Similarly, between April 24 and July 5, 2006, 35 outbreaks of AGE have been reported in 13 cruise ships traveling around Europe. The NoVs have been confirmed in
outbreaks from 9 of 13 cruise ships. Investigation of transmission route are ongoing (364).

1.1.7. **DISEASE TRANSMISSION**

Risk factors such as having a household member with AGE, contact with a person with AGE outside of the household and poor food-handling hygiene are associated with NoV illness. Similarly for SaV, having contact with a person with AGE outside the household increases the risk of infection (141). All these, and many more risk factors are linked by the role that the host, the virus and the environment play in disease transmission.

1.1.7.1. **The role of the infectious agent**

Low infectious doses (231), prolonged asymptomatic shedding (223, 513, 546), environmental stability (31, 41, 156, 444, 557) and great strain diversity (15, 176, 478) increase the risk of infections by this virus family. Moreover, the identification of closely related animal enteric caliciviruses in pigs, and the existence of recombinants within PoNoVs, HuNoVs and also HuSaVs (315, 341, 342) raises concerns for possible human infections or co-infection of animals or humans with human and animal enteric caliciviruses (section 1.1.7.4).

Although several NoV strains circulate and co-circulate in the human population, some strains such as HuNoV GII-4 have emerged as dominant in the U.S. and worldwide. Although HuNoVs belong to genogroups GI, GII and GIV, HuNoV
GII is the predominant genogroup circulating in the population. It is postulated that those strains that cause increased numbers of outbreaks and are widespread worldwide are more virulent and shed in higher titer, or they are more stable in the environment (54, 669). In support, the median cDNA viral load of NoV GII in fecal samples of patients with NoV gastroenteritis is ≥ 100-fold higher than that of NoV GI (3.0 x10^8 and 8.4 x 10^5 copies per gram, respectively), facilitating their transmission (99). If the average number of NoV particles per gram of feces is in this range and we assume that only 0.1% of fecal material (1 mg) is accidentally transferred to a surface, water or food during preparation, the inoculum will contain 3.0 x 10^5 particles. Knowing that the infectious dose for NoV is only 10-10^2 infectious units (231), the inoculum will be enough to cause a new outbreak. 

NoVs show a high frequency of recombination, evidence for positive selection and differential codon use in the capsid region as probable evolutionary mechanisms (589). It has also been proposed that co-evolution between host and pathogen is responsible for their contemporary high diversity in the human population and why new variants spread so rapidly in the presence of human herd immunity (387).

Viruses outside a host could be considered inert particles that can not replicate and eventually most may become inactivated. However some viruses have a degree of “robustness” that allows them to remain infectious for long periods, until a new host is found. This was demonstrated by numerous studies (26, 119, 152, 327, 372, 472, 557) and a high number of viral outbreaks that have been attributable to environmental, water- or foodborne viral transmission (84, 153, 170, 269, 383, 385, 484).
1.1.7.2. The role of the host

Susceptibility to NoVs infection involves genetic factors and acquired immunity. Attempts have been made to define the genetic basis for disease susceptibility. Carbohydrates of membrane glycoproteins or glycolipids are often used by viruses to attach to cell surfaces. The HuNoVs use HBGAs as a receptor. The recognition of human HBGAs by NoVs is a protein-carbohydrate interaction, in which the protruding domain of the NoV capsid (or VLPs) binds to the oligosaccharide side-chains of the HBGAs (628, 629). In addition, a factor in the successful infection of the human intestine by NoVs is the diversity of NoVs and their recognition of different HBGAs present on enterocytes (629). HBGAs are complex carbohydrates present on the surface of red blood cells and the mucosal epithelium of the respiratory, genitourinary and digestive tracts (538). Three major HBGA families are involved in NoV infections: Lewis, secretor and ABO families (387). Secretor negative individuals have nonfunctional FUT2 genes and do not express a fucosyltransferase enzyme responsible for adding a fucose residue to the disaccharide precursor at a \( \alpha 1-2 \) linkage, producing a trisaccharide named H type 1. Instead FUT3, another fucosyltransferase adds a fucose residue at a \( \alpha 1-3 \) or 1-4 linkage producing a trisaccharide named Lewis a (Le\(^a\)). Successive addition of fucose, N-acetylgalactosamine or both to H type 1, will result in tri- and tetrasaccharides named Le\(^b\), ALe\(^b\) and Ble\(^b\) (Figure 1.9) (629). Using Norwalk VLPs as a ligand, it was shown that Norwalk binds to HBGAs of secretors, but not of non-secretors, and also recognizes the A, but not the B HBGAs (286, 294). Secretor negative individuals are also resistant to infection with NoV GII-4 (638), but they will bind VA207, Boxer and OIF VLPs (629).
The binding specificities vary with the NoV strain. Presently, 7 receptor-binding patterns among 14 NoVs have been characterized (287, 629).

Of particular interest is the finding of a specific interaction between the NoV capsid and carbohydrates in oyster tissues (416). The NoVs bound specifically to the digestive ducts of oysters by carbohydrate structures with a terminal N-acetylgalactosamine residue in α-linkage (416). Although carbohydrate-specific antibodies did not recognize the binding site in the oyster tissue, certain lectins such as those derived from Helix pomatia, Dolicho biflorus and Ulex Europeans (that recognize general carbohydrates) had an inhibitory effect on binding, indicating that attachment of NoVs involves carbohydrate structures with a terminal N-acetylgalactosamine residue, similar to the one present in HBGAs. This specific binding could explain why NoVs are not removed from oysters by conventional depuration (416).

Acquired immunity also plays a role in susceptibility to infection, although the mechanism of immunity is still unclear. Short-term resistance to infection occurs following rechallenge with the same strain 6 to 14 weeks later (345) and repeat exposure also seems to lead to resistance to reinfection (324). However, immunity is strain specific, and infection can be developed after challenge with a different strain (704).

1.1.7.3. The role of the environment

In recent years, global population increases, coupled with intensive and increased numbers and confinement of animals on relatively small land areas, have resulted in the generation, accumulation and need for disposal of large amounts of human and animal wastes worldwide (121, 239, 250, 524, 611). The influence that these
changes could have on the transmission of disease to the natural host or transmission of zoonotic diseases via contaminated foods or water sources is now a public health concern (239, 611).

Human enteric viruses can enter the environment through discharge of waste materials from infected symptomatic or asymptomatic people, and be transmitted back to susceptible persons. The best example of this is the fact that human sewage pollution is the ultimate source of virus contaminating shellfish harvesting waters, usually arising from illegal overboard waste discharge from boaters, from failing septic systems along the shoreline or from treated and untreated municipal wastewaters and sludge discharges (585). The impact of sewage on shellfish microbial contamination is not easy to evaluate, but it has been established that shellfish distant from the wastewater input also could be positive by RTPCR (526).

Domestic and industrial wastewaters are collected by a network of sewer lines and are treated at municipal wastewater treatment plants. At the plant, the wastewater undergoes a four step treatment (preliminary sedimentation, primary physical, secondary biological, and tertiary biochemical process). The remaining biosolids material constitutes the sewage sludge (composed of organic matter, human excreta, pathogens, etc) and this must undergo stabilization processing to avoid fermentation, before any kind of application (158). The efficacy of this stabilization process to reduce pathogens varies widely, depending on the pathogen composition (concentration and survival strategies) of the sludge and the process applied (158). Particularly viruses may be absorbed on sludge organic matter and be protected from inactivation (557). For example, studies have demonstrated the presence and persistence of HAV and PV-1 for
at least 3 months in sewage treatment plants, sewage sludge and sludge-amended soil (604, 641). In 1998, 6.9 millions tons of sewage sludge was generated in the U.S.; by 2005 the total increased to 7.1 millions tons and it is estimated that it will increase to 8.2 millions tons by 2010 (524).

Similarly in agriculture, the causative agents of many infectious diseases are excreted by the fecal route, sometimes from clinically healthy animals, and from those with acute and chronic infections. In all types of livestock housing, waste material on site produces odors and contains antimicrobials, nutrients, organic matter and pathogens. As an example, raw swine manure (which contains feces, urine, bedding and water) can contain 100 million fecal coliform bacteria per gram. Approximately 100 million tons of feces and urine are produced by the 60 million hogs raised in U.S. per year (121, 122, 239, 268). With a greater opportunity for horizontal spread of infectious agents among closely confined animals, manure may contain pathogens that can be transmitted to other animals or to workers on the farm or via contaminated water sources.

Different from domestic and industrial wastewaters, storage and treatment of this waste before land application is typically done on site in wastewater lagoons. This system is mainly based on use of anaerobic bacteria (instead of aerobes) to decompose the organic matter, with the advantage that anaerobic lagoons can be deeper, requiring less land area (30). However lagoon management presents significant concerns. Lagoon breaks can release animal waste directly into surface waters or into the surrounding soil and ground water. Heavy rains and flooding can contaminate water supplies as illustrated by the cryptosporidiosis outbreak in Milwaukee (239, 284).
Alternative treatment strategies for animal manure have been developed recently to reduce their impact on the environment and public health (290). On-farm demonstration and evaluation of alternative treatment systems including aerobic systems, solid separation, and chemical treatments are currently conducted (290, 694). To choose the appropriate treatment system for organic waste in agriculture will depend on several factors including reduction of odors, nitrogen, phosphorous and pathogens, resulting product and their potential utilization (683). Because most environmental concerns about waste management have focused on the effects of nitrogen and phosphorous on water or air quality and to bacteria that can be transmitted to humans, several studies have been performed to determine the efficiency of these treatments to reduce their impact on the environment (121, 239, 290, 291, 293, 473, 558, 656). However limited data is available on the efficiency of these treatments to eliminate viruses or their possible surrogates in animal waste.

Several studies have demonstrated the robust survival of viruses in soil, water, food and fomites (557). The longer a virus can survive outside the host, the greater are their chances to find another host. And these chances will be affected by environmental conditions such as pH, temperature, humidity, etc and how they interact with each other and with the viruses. In the particular case of ECV (human or animal), it should be highlighted that because of the lack of a cell culture for virus infectivity, environmental survival of these viruses necessitates assessment through a surrogate virus such as FCV using lab-scale assays. There are two weaknesses to this approach: the first is to understand whether a non-enteric virus, such as FCV adequately reflects the stability of the surrogate enteric virus. A good correlation was observed when FCV and HuNoVs
were inactivated by heat, U.V. or free chlorine, but only HuNoVs remained infectious when the pH was lower than three (149, 156). The second problem resides in the fact that the true environmental scenario can not be fully replicated in a laboratory, because multiple and simultaneous factors affect virus survival in the field.

Different environmental factors affect the fate and transport of pathogens from waste into soil and water. In lab-scale experiments microbial concentrations initially decline with time when added to seawater, but after that they remain basically constant (592). Their studies showed that a 20-fold decrease in FCV infectivity occurs upon addition to seawater, but no significant decrease occurs in the next 24h. However, in the field this also depends on the equilibrium of the microorganism between water and soil and furthermore it depends on the soil composition. This involves the presence of salts, organic matter and pH. Microorganisms exist in a state of zero charge when the pH reaches a characteristic pI and this value varies for each microorganism. Microorganisms with high pI tend to absorb to surfaces to a higher extent than those with low pI. The pI of phage MS2 (pI 3.9) is similar to the pI of HAV (pI 2.8), and lower than the value for PV-1 (pI 7.2). Thus studies performed in 1995 demonstrated that absorption of PV-1 to soil columns was higher than MS2 and HAV (603). The pI of NV (HuNoV GI) determined from VLPs produced in the laboratory was estimated to be 4.9 (539). Based on this value, NV is expected to be more absorptive than MS2, but less than PV-1. The study performed by Meschke and Sobsey in 1998 on the absorption of NV, PV-1 and phage MS2 in 6 different soils confirmed that NV is less absorptive than PV-1 which suggests that it will be easier to remove NV than PV-1 from sediments (447). However prediction of absorptive properties based on pI values refers to overall virus charge under a given
pH, but not to local areas of charge of the virion. Therefore, as demonstrated by Redman et al for MS2 and NV-VLPs, depending on the pH of the environment, viruses with higher pI may display less absorption than viruses with lower pI (539).

It is a general concept that very low or high pH may decrease pathogen viability. However in the case of NoVs it has been demonstrated that pH lower than three or higher than ten will not affect virus stability (156). Moreover evidence suggests that adsorption of viruses to particulate matter and sediments confers protection against the inactivating influences of pH. Solar radiation promotes inactivation of viruses through visible and short wave U.V. components. Again lab-scale experiments showed that although differences exist between U.V inactivation of surrogates of NoVs (FCV and CaCV) in suspensions or on dried surface, inactivation is also achieved in the presence of high organic material because RNA is the target. If U.V. is compared with ionizing radiation such as gamma rays, the former is more effective in the presence of solutes that can react with free OH radicals (139). However, in contrast to studies of water sanitation, the effects of these radiations have not been extensively studied for animal wastes.

Viruses may be released from the host in an aggregated state. In general it is assumed that aggregated viruses are more resistant to inactivation than single virus particles, because each virus particle within the aggregates must be inactivated before the whole aggregate is considered inactivated. Adsorption to sediments may decrease because of the aggregated state, which could be beneficial in terms of limiting local soil contamination, but these particles can easily be transported by the air or rainfall to other points. Virus-like-particles as laboratory surrogates for enteric caliciviruses have been used to assay reduction of NoVs in soil columns under different conditions that resemble
those in the field (different soil composition, groundwater and rainwater) (447). Although these experiments and results can be extended to estimate and understand what hypothetically would happen in the field, the true scenario where all the above mentioned factors interact has to be evaluated.

If viruses can reach the water through overland flooding, illegal discharge or partial treatment of animal or human waste, the risk of water-borne outbreaks is increased, because as demonstrated by studies with surrogates, viruses are able to survive in seawater, fresh water, ground water and drinking water and also are resistant to inactivation treatments (557). Current water quality standards are based on the concentration indicator microorganism (fecal coliforms level) and the intended use of the water (drinking, irrigation, livestock watering or recreational). This adds a new variable, because as it was demonstrated, the correlation between fecal coliforms level and virus presence and concentrations is not accurate. Global challenges in water, sanitation and public health include, among others, contamination of water in distribution systems and the potential for water reuse and conservation (457). The latter is defined as the use of highly treated wastewater for irrigation or landscaping (non-potable reuse) or to supplement surface or groundwater sources used as drinking water supplies (potable reuse). However, this will be viable only when a careful, thorough project to monitor contamination, health risks and safety factors exists (467). If contaminated water is accidentally used for crop or vegetable irrigation, for growing shellfish, or recreational purpose (lakes, swimming pools, etc) waterborne outbreaks are inescapable (272, 456, 590).
1.1.7.4. **Risk of zoonotics**

The relationships between human and animal hosts for ECV are unclear and are under investigation. On the one hand, the existing PoSaVs as well as BoNoVs belong to different genogroups than HuNoVs and HuSaVs, and it is unlikely that they may represent a risk for humans (254, 497). However, antibodies to BoNoV GIII were detected in the human population. Twenty-two percent of the sera tested had IgG antibodies to BoNoV GIII VLPs. Although there was some cross-reactivity between BoNoV GIII and HuNoV GI, sera showed high reactivity to BoNoV and low reactivity to HuNoV GI suggesting a specific response. Seroprevalence was also significantly higher among veterinarians when compared to the rest of the population, suggesting a higher risk of exposure (687). These results suggest that humans may become infected with bovine-like NoVs, although the infections might be asymptomatic.

On the other hand PoNoVs belong to GII, the most frequently detected genogroups in humans, suggesting a potential risk of related strains for humans (675). Studies of the seroprevalence and antigenic cross-reactivity between porcine and HuNoVs, also support this possibility. In a survey of antibodies against SW918 (a PoNoV GII strain detected in Japan), the authors reported that 83% of sera from domestic pigs in the U.S. showed positive reactions and 52% reacted with HuNoV GII. Surprisingly, 63% of sera also reacted with the NV strain, a HuNoV GI (174). Viruses in these two genogroups do not cross-react and no PoNoV GI has been detected in swine. Recently, it was observed that convalescent serum from Gn pigs inoculated with PoNoV GII, cross-reacted with VLPs of several HuNoV GII strains, but not NoV GI VLPs, confirming the antigenically similarly between GII NoVs in pigs and humans (675).
addition to this, HuNoV replication in Gn pigs was confirmed by serial passage of HuNoV in seronegative Gn pigs, with occurrence of mild diarrhea and shedding, and detection of structural and nonstructural proteins in enterocytes (106).

Outside of the NoV and SaV genera, but still within the *Caliciviridae* family, the Vesicular exanthema swine virus (VESV), is an example of a marine calicivirus causing disease in terrestrial species (600). The first outbreak was traced back to California in 1932, when swine and cattle became sick with vesicular lesions after being fed with raw garbage collected from restaurants (598). This and successive outbreaks until 1952 were controlled and many different, but related VESV strains were detected. In 1952, the disease “traveled” from the West coast to the North central U.S. and in 14 months all swine growing areas in the U.S. had reported VESV. The source of infection was raw Californian pork trimmings discarded in Wyoming and latter used to feed swine. In 1959 the disease was eradicated. (598). In 1972, a calicivirus that causes classical VES in swine was isolated from a pinniped and named San Miguel sea lion virus type 1 (SMSV-1) (596). Several years latter, many similar viruses were detected in different species of pinnipeds and cetaceans of the north Pacific Ocean, as well as in ocean fish (the opaleye perch) (601). By this time, antibodies to different SMSV serotypes were reported in humans in the U.S. (599), leading to the conclusion that fish were the vehicle for the cross-species infection. The extent of the human disease was suggested by seroprevalence studies of Vesivirus in humans (597). The prevalence of antibodies against SMSV ranged from 12-47% among 765 sera from blood donors and patients with clinical hepatitis of unknown etiology. Additionally viremia was detected in 9.8% of 112 sera tested by
RTPCR. Thus this information suggests that interspecies transmission among members of the *Caliciviridae* family is possible and require continuous investigation.

### 1.1.8. IMMUNITY

#### 1.1.8.1. Immune response to natural infection in humans

Immunity to HECV is not completely understood. Again, because of their high impact in the population, most efforts are focused on understanding the immune response to HuNoVs. Studies of human volunteers showed that not all individuals are susceptible to NV infection and/or disease, short-term immunity is apparently conferred by repeat exposure, but immunity is strain specific (324, 508, 704).

Susceptibility to disease depend on two factors: acquired immunity and HBGAs (405). Studies by two different groups have found that serum antibody titers higher than 1:100 were associated with protection against NV infection (51, 555). However this was not the case in studies of adult volunteers by Blacklow et al (52), where ill volunteers challenged with NV had high antibody titers. Studies by Parrino et al (508), found that 6/12 people challenged with NV developed disease, whereas another 6 remained asymptomatic. One year later, when the same 12 people were rechallenged, those that were ill one year before, became ill again, whereas the asymptomatic remained asymptomatic. This demonstrated that long term immunity did not exist and that other factors were involved in susceptibility.

In naturally occurring outbreaks, NoV specific IgG and IgA were evaluated in adults. Both homologous and heterologous antibody responses were elicited after NoV infection and an increase in antibody avidity occurred for specific and cross-reactive
antibodies, at least within GII (545). Similar results were observed by Farkas et al (175) among crew members after an AGE outbreak on U.S. Navy vessel.

Studies of volunteers inoculated with the GI NV strain showed that those who had nonfunctional FUT2 (non-secretors) were resistant to infection. The same study also showed a correlation between protection from NV infection and the presence of a memory sIgA response in uninfected susceptible volunteers. Moreover, some susceptible volunteers that were not infected did not have sIgA responses, indicating that sIgA and non-sIgA related mechanism are involved in immunity to NoVs (405).

In another study, the same authors evaluated cellular and humoral immunity following challenge with GII SMV strain. In contrast to NV, SMV infection was not dependent on known HBGA status. Sixty percent of volunteers showed 4-fold or more increased IgG antibody titers in serum and salivary IgA antibodies. Significant increases in serum IFN-γ and IL-2, but not IL-6 or IL-20 were detected on day 2 post challenge. IFN-γ production was dependent upon CD4 cells, indicating a predominant Th1 response (404). Similarly both IL-2 and IFN-γ levels were significantly increased in NoV-associated diarrhea specimens from travelers with diarrhea due to NoV, suggesting a dominant Th1 immune response (357).

1.1.8.2. Experimental studies in animal models

Several attempts have been made to study immune response to NoVs in different animal models, with variable results. After experimental inoculation of chimpanzees with NV, significantly increased serum antibodies against NV in all animals occurred and viral antigen was detected in feces of 5/9 (705). However clinical illness did not occur. Disease
transmission was demonstrated by feeding another 4 animals with fecal filtrates from the first experimental group. Again antibody titers increased in serum and viral antigen was detected in 50% of the animals in absence of clinical signs. Similar studies of newborn pigtail macaques inoculated with GII Toronto strain, also showed viral RNA in feces together with clinical signs (620). Neither clinical signs, nor antibodies responses were observed when common marmosets or cotton top tamarins were inoculated with HuNoVs. Short term viral shedding was detected in fecal samples of cynomolgus macaques. In contrast 1/4 Rhesus macaque shed virus and developed NV specific IgM and IgG response (547).

Antibody responses were also investigated in BALB/c mice given NV VLPs. To evaluate induction of mucosal antibody responses, recombinant NV-VLPs were administered to BALB/c mice by PO and IN routes. Low doses of VLPs were more immunogenic when administered IN than PO in the absence of adjuvant, and addition of the adjuvant [mLT(R192G)] enhanced the magnitude and the duration of the Ab response (28, 240). Similarly, HuNoVs GII VLPs induced high serum as well as fecal IgA Abs when administered alone by the IN or PO route. Co-administration of adjuvant resulted in higher Ab titers. A Th1/Th2-like cytokine profile was observe in cervical lymph node and Peyer’s patch cell cultures, independent of the route or adjuvant (474).

1.1.9. CONTROL AND PREVENTION

1.1.9.1. Preventive strategies

Knowing the mechanism of spread/transmission and survivability of NoVs has helped to develop guidelines for prevention and control of disease. Interruption of
transmission is the primary strategy of control. The interventions used depend on the mode of transmission and the source of contamination, but an early detection and rapid response are keys to control outbreaks. In the case of hospitals, infection control efforts must prioritize the prevention of spread of infection to other clinical areas by containment of infected/exposed individuals (especially the prevention of patient and staff movements to other areas), hand-hygiene and effective environmental decontamination (97). Similarly in day care centers and nursing homes, people with AGE must be treated as suspected NoVs AGE, and avoid contact with others. Attention should be given to hand hygiene and disinfection of surfaces that can be contaminated (301). Practices to avoid viral contamination, recontamination and survival during food processing are defined under the Good Manufacturing Practices (GMP), Good Hygiene Practices (GHP) and Hazard analysis and critical control points protocols in processing of food, particularly seafood (292). Basically it is very important: (i) to clean any surfaces with appropriate disinfectants, (ii) for food handlers to maintain strict personal hygiene; (iii) to use hand antisepsis and (iv) for ill workers to be excluded from food handling for several days after the end of the symptoms (49). In the particular case of seafood, consumption of raw shellfish is a risk, because they are harvested from areas that met sanitary standards that are based on bacterial microbial levels, which may not be appropriate for viral contamination of shellfish or because of washing with contaminated water. Foodborne illness can be prevented by thorough cooking. Sometimes all, part or more than these prevention and control measures must be applied together to manage outbreaks of unexpected dimensions (95).
1.1.9.2. **Antivirals**

Several points where replication of caliciviruses, particularly NoVs could be inhibited have been proposed. However most, if not all can not be assayed until a routine continuous cell culture system is developed. For example, the carboxyl terminal segment of the RdRp lies in the active site of the enzyme and it plays a role in RNA stabilization. Drug compounds that specifically disrupt the interaction between the carboxyl terminal segment and the active site cleft may interfere with viral replication (470). Initiation of translation by members of the *Caliciviridae* family involves the interaction of VPg with eIFs, like other viruses, but particularly it requires the RNA helicase component eIF4A, because the viral NTPase lacks this activity (105). Compounds that inhibit eIF4A may also represent good antivirals.

Antisense and RNA interference (RNAi) technologies could also be applied. Antisense RNA therapy mediated by phosphorodiamidate morpholino oligomers (PMO) targeting ORFs was assayed for Vesiviruses. The PMOs contain purine or pyrimidine bases attached to a backbone composed of 6 morpholine rings joined by phosphorodiamidate intersubunit linkages. They bind to mRNA and prevent translation by steric blocking. Porcine kidney and African green monkey kidney cells were infected with Vesivirus strains SMSV-13 and primate calicivirus PAN-1. Different PMOs with sequence complementary to the AUG translation initiation site of ORF1, ORF2, and ORF3 were evaluated for their effects on viral titers. Various PMO sequences to an upstream region of ORF1 were effective in reducing viral titers up to 80% in a dose and sequence dependent manner (613).
The silencing effects of RNAi are highly specific and only require that the sequence of the target RNA be known. The mechanism includes a long dsRNA processed to short interfering RNA (siRNA) by the action of dsRNA-specific endonuclease (DICER). The resultant siRNA are 21-24 nt dsRNA with a 3’ overhang of 2 nt. They are incorporated into a nuclease complex known as RNA-induced silencing complex (RISC), ATP unwind and the complex is directed by the unwound antisense siRNA to the homologous target RNAs which undergoes endonucleolytic cleavage (614). In treatment of infectious disease, the target RNAs are exogenous and can be inhibited without affecting the host. Several studies have shown that RNAi can directly target viral transcripts (209, 388, 479) or in the case of RNA viruses, the viral genome itself (120, 306).

1.1.9.3. Vaccines

Currently no vaccines for NoVs exist. Vaccination is a cost-effective approach to reduce morbidity and mortality in a population. The rationale to develop a vaccine is based on the clinical significance of a specific disease in the general population or in a specific group (166). Epidemics of NoV AGE affect people of all ages, but particularly large groups in closed environments, such as nursing homes, day care centers, schools, travelers and the military. So an efficacious, cost effective vaccine will be useful in these situations.

However there are a number of obstacles to development of a NoV vaccine: (i) the lack of a routine cell culture system in which NoVs can be propagated. A new 3D cell culture system recently developed could be a breakthrough, but the capacity to produce large volumes and high titers routinely is not yet established (615). Because of this, NoVs
can not be propagated to high concentrations and large volumes as a source for live or inactivated vaccines; (ii) lack of in vitro cell culture precludes analysis of the role of neutralizing antibodies in vaccine protection; (iii) additionally immunity against NoVs is not clearly understood and other genetic host factors, besides immunity play a role in susceptibility (see section 1.1.7.2 and 1.1.8).

With the development of VLPs, a potential opportunity for the design of a NoVs vaccines emerged (319). Several laboratories have focused studies using this approach. Preclinical testing of a candidate NV-VLP vaccine was conducted in mice. NV-VLPs were immunogenic when orally administrated with or without the adjuvant cholera toxin (CT). Systemic and mucosal IgA Abs were detected in the absence of CT, and the presence of CT resulted in increased IgA Abs titers. IgG Abs in serum were detected 9 days post vaccination and fecal IgA Abs at 24 days post vaccination (28). Studies of volunteers demonstrated that NV-VLPs are immunogenic when orally administered. Serum IgG Ab responses were dose dependent, and all vaccine given 250 µg of VLPs responded with 4-fold or more increases in serum IgG Ab titers. No increases were observed after a second VLP dose (27).

Healthy adult volunteers developed significant rises in IgA antibody secreting cells (ASC) after receiving NV VLPs orally. About 30-40% developed mucosal IgA ASC, and only the group that received 250 µg (but not higher doses) showed increased IgG ASC in serum. Transient lymphoproliferative responses and IFN-γ production were observed in those who received 250ug or 500ug (but not higher) VLP doses (627). A genetically modified cholera toxin, CT-E29H was evaluated as adjuvant for NV-VLPs in BALB/c mice. A low dose of NV-VLPs with this adjuvant delivered by the IN route was
more effective than the highest dose delivered by the oral route, at inducing both cellular and NV-VLPs specific IgG and IgA Ab responses (519).

Because VLP do not replicate in the host, large quantities in multiple doses may be needed for vaccine. To achieve this, a subunit vaccine was developed in plants (potatoes) (626) and also Venezuelan equine encephalitis replicon as a vector to express the capsid protein in mammalian cells has been used (263).

1.2. **ROTAVIRUS**

1.2.1. **INTRODUCTION**

Human rotavirus (HRV) (70nm in diameter) was first discovered by Bishop et al in 1973 and it was associated with severe endemic diarrhea in infants and children (50). Like NoVs, the discovery was based on EM but in this case using transmission EM of duodenal mucosa. Soon after that, rotavirus (RV) was identified by many others in feces by negative stain EM (181, 335, 451). It became clear that RV was an important etiological agent of diarrhea in infants and young children causing 35\% to 50\% of the hospitalizations during the first 2 years of age (333).

1.2.2. **INFECTIOUS AGENT**

1.2.2.1. **Agent**

Rotaviruses belong to the *Reoviridae* family and posses a segmented dsRNA genome consisting of 11 segments. Rotavirus particles consist of triple-shelled capsids
with two viral proteins, VP4 and VP7 comprising spike and the outer capsid, respectively, one inner capsid (VP6) and the core (VP2) layer. The term RV is derived from the Latin word “rota”, which means “wheel”, suggested by the sharply circular outline of the outer VP7 capsid, giving the appearance of the rim of a wheel placed on short spokes radiating from a wide hub (164).

1.2.2.2. Taxonomy

Classification and cross-protection between strains of RV are based on the outer capsid proteins, VP4 and VP7. The VP7 (major outer surface component) is a glycoprotein whereas VP4 or rotavirus surface spike, is a protease-sensitive protein. The glycoprotein (G) type is defined by VP7 whereas the protease sensitive (P) type refers to the VP4 protein (334, 561). For RV-A, 15 G serotypes/genotypes (G1-G14) have been identified. The G-types 1 to 6, 8 to 10 and 12 were isolated from human infections, whereas the main G-types in pigs are 3, 4, 5 and 11 (334, 561, 651, 697, 716). To date, at least 11 P serotypes (determined by neutralization assay with polyclonal or monoclonal antibodies) and 22 different P genotypes (determined by hybridization and sequence analysis) have been described for RV-A (278, 279, 406, 432, 489). Because a complete correlation between P serotypes and P genotypes does not exist, a different designation has been adopted with open numbers for P serotypes, and numbers between brackets for P genotypes. Among human rotavirus (HRV) strains, P1, 2, 3, 4, 5, and 11 were detected, whereas P2 and 9 were detected in pigs (167, 281, 432). Among the 22 P genotypes identified, P[4], P[6], P[8], P[9], P[10] and P[12] are associated with HRV and P[6], P[7], P[14] and P[19] with pigs (75, 220, 285, 561, 716, 718). For HRV P[4], P[6], P[8] and P[9] correspond to serotypes P1B, P2A,

1.2.2.3. Genome organization

The 11 dsRNA segments encode six structural and six non-structural proteins (334, 561, 716). The 11 segments of several strains have been full sequenced. The prototype simian SA-11 strain was the first complete genome sequenced. Each positive-sense RNA segment starts with a 5’-NTR, followed by at least one ORF coding for the protein, and the 3’NTR that includes a subset of conserved sequence and ends with two 3’terminal cytidines (164). Almost all mRNAs end with the consensus sequence 5’-UGUGACC-3’, containing signals for gene expression and replication. The length of the 3’ and 5’-NTR vary for different genes, and no poly (A) tail is found at the 3’ end. All sequenced segments possess at least one ORF after the first AUG. Genes 7, 9 and 10 contain a second in-phase ORF and gene 11 has a second out-of-phase ORF. All genes are monocistronic, except gene 11 (435). The dsRNA are base-paired end-to-end, and the positive-sense strain contains a 5’cap sequence m7GppG(m)GPy (435). Packaging of these segments into the capsid requires protein-RNA interactions (331).

In general, segment one, two, three, four, six and nine encode structural proteins VP1, VP2, VP3, VP4, VP6, and VP7, respectively, whereas segments 5, 7, 8, and 10 encode non-
structural proteins NSP1, NSP3, NSP2, NSP4, respectively, and segment 11 encodes NSP5 and NSP6. The VP7 is encoded by segment 7, 8, or 9 depending on the strain.

1.2.2.4. **Virus structure**

The viral genome is contained within a triple layered capsid, which also contain the virus RdRp required to transcribe the individual RNA segments into active mRNA. The numbering of the structural proteins is based on their molecular weights, with VP1, the largest at 125 kDa, and VP8, one of the two proteolytic products of VP4, the smallest at 28 kDa. The six structural proteins form the multilayered capsid of the mature virus particle, whereas the non-structural proteins (NSP), with the exception of NSP1, are essential for replication.

**VP1:** All VP1 contain the 4 conserved motifs present in all RdRp. The VP1 binds nucleotides and specifically binds to the 3’ end of viral mRNA. These and other factors, make VP1 the viral RNA polymerase that functions as a viral transcriptase and replicase. However, VP1 requires VP2 for replication activity (514). Structural data for RV and other dsRNA viruses shows that each genome segment is spooled around a transcription complex (VP1/VP3) that is anchored to the inner layer of VP2 (216).

**VP2:** Removal of VP6, results in single layered particles composed of VP2. The VP2 encompasses the dsRNA, within a protein layer of 120 monomers arranged in a T=1 icosahedral lattice(377). Although VP2 binds RNA non-specifically (65, 374), it is essential for replicase activity together with VP1, and its role in replication is linked to its ability to bind mRNA (164). Cryo-EM analysis demonstrated that the VP1/VP3 transcription enzyme complexes are attached to VP2 and surrounded by ds RNA (378).
VP3: It is a guanylyl and methyl transferase, which functions as a multifunctional capping enzyme (107, 410). It also binds ssRNA and interacts with VP1 to form the transcription complex.

VP4 (VP5 and VP8): The outer capsid of the triple layered particle contains 60 spikes, each of which is formed by a dimer of VP4. The VP4 spikes are non-glycosylated and exhibit two distal globular domains, a central body, and an internal globular domain that is tucked inside of the VP7 layer in the peripentonal channel of the T=13 (see VP7) (583, 708). Among its functions, VP4 is a hemagglutinin, neutralizing antigen, cell attachment protein and contains a putative fusion region (164). Proteolytic (trypsin mediated) cleavage of VP4 into VP5 and VP8 results in enhancement of viral infectivity. The VP5 and VP8 products are analogous to the cleaved products of the influenza virus hemagglutinin, HA1 and HA2. Much like VP8, HA1 plays an accessory role by providing initial binding to cells via sialic acid containing receptors, whereas HA2, more like VP5, it is sufficient on its own for cell fusion (691).

VP4 induces neutralizing antibodies and antibodies against VP4 neutralize RV in vitro (78, 128, 234, 280, 355, 631), and passively protect mice against heterologous challenge in vivo. In animals, VP4 induces protective immunity (425, 487) and it is immunogenic in children and animals (124, 584, 623).

VP6: The intermediate layer of the triple layered particle is formed by VP6, and it is in direct contact with VP7. Particles that have lost VP7 and VP4 are called double layered particles and they maintain the same icosahedral symmetry as VP7. The VP6 is the major protein of RV by weight. It plays a role in the overall organization of the
particle by interacting with the outer layer VP7 and VP4 and the inner layer protein VP2, integrating cell entry and endogenous transcription (521). The assembled particle is very stable and has conserved epitopes that are target for diagnosis. The VP6 which is the basis to classify rotaviruses into seven serogroups (A-G) (560, 561).

**VP7:** The major component of the outer layer of the triple layered particle and second most abundant protein is VP7. Seven hundred eight copies of VP7 are grouped as 260 trimers at all the icosahedral and local three-fold axes as a T=13 icosahedral lattice surrounding 132 channels. The 132 channels are grouped into three classes: (i) 12 type I channels located at the five-fold vertices of the capsid; (ii) 60 type II channels at each of the pentavalent locations surrounding the type I channels, near which VP4 is attached to VP7 and VP6; and (iii) 60 type III channels located at the remaining hexavalent positions on the capsid surrounding the icosahedral three-fold axes (521). The VP7 is highly immunogenic and induces neutralizing antibodies (334, 561, 716). This protein contains only N-linked high-mannose oligosaccharide residues that are added cotranslatinally as this protein is inserted into the ER. This is a glycoprotein in most strains although glycosylation is not requires for capsid assembly (164).

**NSP1:** This protein is a RNA-binding protein that directly interacts with IRF-3. It plays a role in pathogenesis in some animal models, by antagonizing the type I IFN response to increase viral pathogenesis (221, 222).

**NSP2:** It is a highly conserved basic protein, expressed at high levels in infected cells. It has NTPase activity, but it is also considered very important for replication and packaging. Proof of this are the fact that NSP2 accumulates at the genome replication
site (viroplasm), it is a component of the replication intermediates, with replicase activity (515) and if interacts with the viral RdRp (343).

**NSP3:** It is involved in specific recognition of viral RNA, facilitating translation by the cellular machinery. NSP3 is a functional homologue of cellular poly (A) binding protein. The N-terminal domain of NSP3 interacts with the 3’ consensus sequence of the viral mRNA, whereas the C-terminal domain interacts with the eIF4G to facilitate circularization of viral mRNAs and its delivery to the ribosomes for protein synthesis (521).

**NSP4:** NSP4 is an endoplasmic reticulum-specific glycoprotein that functions as an intracellular receptor by mediating the conversion of double layered to triple layered particles in the endoplasmic reticulum. In addition this protein is reported to have enterotoxicity activity. The NSP4 may interact with a cellular receptor of the gut epithelium and thereby stimulating a calcium-dependent signal transduction pathway that increases plasma membrane chloride permeability and potentiates chloride secretion, inducing secretory diarrhea (334).

**NSP5:** This protein is acidic and rich in serine and threonine residues. It forms dimers (527), binds ss and dsRNA (659), and undergoes phosphorylation and O-glycosylation in the infected cell (682). The accumulation of NSP5 in the viroplasm, suggests that the protein is involved in genome replication or core assembly. However other evidence indicated that perhaps its RNA binding activity is connected to suppressing the dsRNA-activation of the antiviral response such as protein kinase PKR by sequestering viral RNA generated within the viroplasm (682).

**NSP6:** It is a component of the viroplasm and interacts with NSP5 (521).
1.2.2.5. **Physicochemical and biological characteristics**

Rotaviruses are non-enveloped viruses, resistant to inactivation by ether, chloroform, detergents, many chemical disinfectants and antiseptics (1). However phenols, formalin, chlorine and ethanol (95%) have been shown to be effective (568, 716).

**Chlorine:** Chlorine is considered among the most effective disinfectants against RV in drinking water and wastewater. Several studies have demonstrated that chlorine inactivation is dose, pH, exposure time and virus type dependent (3, 488, 658). The inactivation of simian rotavirus SA-11 and HRV Wa by chlorine was compared at 4°C at different pHs and doses by Vaughn et al (658). The two viruses did not show significant differences in their behavior. Both viruses were usually more readily inactivated at pH 6.0 than at pH 8.0 when low chlorine concentrations (0.05 to 0.2 mg/liter) were used. A complete (5 log₁₀) reduction of both was obtained within 20s at all pH levels when chlorine concentrations were increased to 0.3 mg/liter. Little inactivation was observed when copper and silver ions, in combination with low levels of free chlorine were assayed in water (3). In their studies of RV inactivation, Ojeh et al (488) evaluated the efficiency of chlorine on infectivity and its correlation with the presence of viral RNA. The authors observed that 2,500 ppm chlorine completely destroyed the infectivity of RV as well as viral RNA amplifiable by PCR.

**U.V. and Gamma radiation:** U.V. inactivation has been shown to be effective for inactivation of RV (34, 488, 595). The irradiation completely destroyed the infectivity of RV as well as viral RNA amplifiable by PCR (488). The effects of U.V. irradiation were also evaluated (34) with RV in phosphate buffered water. The 99.9% inactivation dose for SA-11 was 42 mW sec/cm², almost three times higher than the dose required for
achieve 99.9% inactivation for hepatitis A. However all of these studies have been done using virus in buffered solutions, and the influence that the presence of solid organic material may have such as in fecal suspensions or food, has not been evaluated.

**Temperature:** Survival and inactivation of RV have been studied at different temperatures and under different substrate compositions (buffered solutions, food and feces) (40, 536). The SA-11 RV infectivity remained after incubation for 1h at 37°C, 24-48 h at 25°C or 5 min at 50°C. In addition it was also stable after milk pasteurization (15s at 80°C) (40, 334).

Rotavirus also maintained its infectivity for 7-9 months at room temperature (18-20°C). Ramos et al (536) analyzed the stability of porcine rotavirus (PoRV) in feces. Fecal samples were kept at 10°C without any preservative for 32 months. After that, the integrity of viral RNA was demonstrated by polyacrylamide gel electrophoresis (PAGE) and RT-PCR and correlated with virus infectivity by inoculation of MA-104 cells. Thirty-percent of these samples were still positive by PAGE after 32 months. Fifty-percent of these PAGE-positive samples retained infectivity in cell culture and 60% were positive by RT-PCR.

**pH:** Rotaviruses are stable at a pH range from 3 to 9 and infectivity is relatively stable within this pH range (334, 716). In vitro, initial binding is sodium dependent, pH insensitive between 5.5 and 8.0, and dependent on sialic acid residues on the membrane (334). However pH indirectly affects inactivation by other methods.

**Other methods:** Several other methods have been tested for inactivation of RV (110, 347, 529, 670). The non-ionic disinfectants, sodium hypochlorite and formaldehyde did not inactivate RV in feces, whereas 95% ethanol was effective. Infectivity of SA-11
RV for MA-104 cells was maintained after treatment of RV with organic solvents, repeated freezing and thawing cycles and sonication (334).

An interesting approach for inactivation was proposed by Pontes et al (529). The authors showed that inactivation of RV by high pressure was effective without loss of immunogenicity based on neutralization titer in plaque reduction assays, antigen titer in ELISA and direct interaction with the particle, as measured by gel-filtration chromatography. After pressure treatment, the particles were recovered with slight structural changes in VP4 compared to urea denaturation and controls.

Another alternative development was a pilot study conducted to determine if bacterial proteases could inactivate RV. Alcalase was the most effective among several proteases tested. However the results showed that this protease was able to inactivate RV to a certain degree depending on pH, temperature and protease concentration. At pH 6.0 and 25°C, (similar to field conditions) 1% alcalase reduced the SA-11 RV titer by 2.75 log₁₀ in 24h, and by 3.25 log₁₀ in 120h. However, it is necessary to investigate inactivation under field conditions where several substrates can compete for the same enzyme (670).

The inactivation of HRV Wa and simian RV SA-11 by chlorine dioxide was investigated at 4°C in phosphate-carbonate buffer at pH 6.0 to 8.0. Both viruses were rapidly inactivated, within 20s under alkaline conditions (pH 8.0), with chlorine dioxide concentrations ranging from 0.05 to 0.2 mg/liter. Similar reductions of infectivity required additional exposure time of 120s at pH 6.0 and inactivation was moderate at neutral pH (110). Kawana et al (347) studied the inactivation of a range of enveloped and non-enveloped viruses, by povidone-iodine (PVP-I) and other commercially available
antiseptics in Japan. RV was inactivated by PVP-I drug products, benzalkonium chloride and benzethonium chloride within a short period of time, but it was not inactivated by chlorhexidine gluconate or alkyldiaminoethyl-glycine hydrochloride.

1.2.3. DIAGNOSIS

Rotavirus can be detected in feces of infected people or pigs by several techniques such as RT-PCR, Nested or seminested- polymerase chain reaction (Nested- or seminested-PCR), PAGE, electron microscopy (EM), immune electron microscopy (IEM), immunofluorescence (IF), virus isolation (VI), latex agglutination (LA), and ELISA (303, 561, 716).

1.2.3.1. Antigen detection: classical techniques

1.2.3.1.1. EM and IEM

Methods such as EM or IEM with a sensitivity of $10^5$ to $10^8$ viral particles/ml (499) provide additional important information on morphology of viruses or presence of other viruses. The advantage of the EM or IEM is the high specificity, because of the virus distinctive morphology. Direct EM allows detection of RV in 80% to 90% of the virus positive specimens (67).

1.2.3.1.2. ELISA

During an acute rotavirus infection, approximately $10^8$ to $10^{12}$ viral particles/ml are excreted. In these circumstances, diagnosis by ELISA (sensitivity of $10^5$ to $10^6$ viral
particles/ml) (205, 552) and rapid tests, such as LA with a sensitivity of $4 \times 10^6$ up to $2 \times 10^7$ infectious particles/ml fecal suspension are the methods of choice in many laboratories. Commercial kits for detection and serotype determination are available in ELISA format.

1.2.3.1.3. Monoclonal antibodies

Enzyme-linked immunoabsorbent assay with mAb to RV-A Vp6 protein also showed higher sensitivity than EM (22.2% and 13.4%, respectively) to detect RV in fecal samples of piglets affected with diarrhea (548). The authors also indicated that some samples were positive by EM but not by ELISA-mAb. It was suggested that these samples were RV-C, showing that although EM is less sensitive, it was able to detect a wider range of RV. Monoclonal Ab directed to different epitopes of the same serotype are necessary because of the epitope polymorphism within a serotype (129, 159). This mAb together with one or more polyclonal antisera have been employed in ELISA sandwich format for RV serotyping.

1.2.3.1.4. Cell culture

However none of the techniques mentioned earlier differentiate between infectious or non-infectious virus, which is very important to assess the risk of disease transmission from environmental samples. Human RV were first adapted to grow in an African green monkey kidney cell line (MA-104), and porcine RV was first adapted to grow in primary porcine kidney cell culture (637) and latter to MA-104 in roller tubes (57). Addition of proteolytic enzymes is essential for virus propagation. Virus activation
requires addition of 10µg/ml of trypsin or 2.5µg/ml of pancreatin before infection. Cytopathic effects are characterized by rounding of cells followed by cell detachment from the monolayer. Viral antigens can be detected by immunofluorescence or immunochemical staining in the cytoplasm of the infected cell (716). Group C and B porcine RV have also been propagated in cell culture using roller tubes and high pancreatin concentrations (565, 636). The porcine RV Cowden strain (RV-C) has been propagated in MA-104 cells (562).

In general, the detection limit for ELISA is 10⁵ RV particles/ml, and RTPCR assays detect RNA from 10¹⁻³ particles/ml. Cell culture propagation has been shown to detect 10⁰⁻¹ RV infectious particles/ml that does not differ from RTPCR and could be used as a substitute technique, but it is more time consuming and requires maintenance of cell lines. Neutralization antibodies are detected by plaque reduction (the most sensitive technique), neutralization of IF foci, neutralization of virus as determined by quantitation of viral antigen by ELISA, or inhibition of CPE in roller-tube cultures (333).

1.2.3.1.5. PAGE

The 11 RNA segments of RV fall into 4 size groups. The distribution of the segments into 4 groups is shown by polyacrylamide gel electrophoresis (PAGE) and the number of segments in each of these 4 groups is characteristic of each A-G RV group. Electrophoretic pattern of RV group A shows 4 high molecular weight dsRNA segments (1-4), two middle size segments (5-6), a distinctive triplet of segments (7-9), and two smaller segments (10-11) (Figure 1.10). When this pattern is not observed, the sample under analysis may be (i) an avian RV-A, (ii) a non-group A, characterized by a 4:2:2:3
or 4:3:2:2 arrangement for RV-B and RV-C, respectively, or (iii) a RV-A with rearrangement within individual segments. The PAGE (sensitivity of $8 \times 10^8$ to $8 \times 10^9$ viral particles/ml) (499) is a relatively easy technique to detect and differentiate RV from reoviruses and to identify RV groups (308). However serological and genomic confirmation is required to confirm RV group and serotype.

1.2.3.2. **Nucleic acid detection: molecular techniques**

1.2.3.2.1. **RTPCR, nested-, seminested-PCR and multiplex**

Although all of these tests have shown high specificity and sensitivity, in the case of delayed sampling or environmental samples where viruses are not replicating, the amount of virus is usually under the detection level for these techniques. In this scenario, molecular techniques or techniques that combine molecular with virus replication approaches are need. The sensitivity and specificity of several techniques were compared. The sensitivity of seminested-PCR (100%), ELISA (100%) and EM (95%) was higher than LA (68%) and RT-PCR (58%) for fecal samples (431). The RT-PCR, nested-PCR and seminested-PCR techniques have been developed to detect RV-A, RV-B, and RV-C and also to differentiate RV-A G and P-types (217, 219, 220, 535, 662, 697). To compare cell culture and nested-PCR, the presence of RV in cell culture and fecal samples was assayed. The detection limit for virus in cell culture supernatants was $3 \times 10^{-2}$ TCID$_{50}$ by RT-PCR and $3 \times 10^{-3}$ TCID$_{50}$ by nested-PCR (161).

For environmental samples, most studies have focused on detection of RV in water as a source of infection. The critical point in this type of sample is to concentrate the virus and for that several methods have also been assessed (4, 68, 83, 224, 282, 356,
After concentration is achieved, detection of RV has been performed by RT-PCR, seminested-PCR, and recently flow cytometry (FC) (2, 62, 185, 655).

1.2.3.2.2. **Real-time:**

Real time RTPCR has been introduced for diagnosis of RV in fecal samples but mainly in environmental samples. When rt-RTPCR, a conventional nested-RTPCR and EM were compared, RV was detected in 123 samples (20%) with the rt-RTPCR, 113 samples (18%) with the nested-RTPCR assay, and 79 samples (13%) with EM. Using serial diluted nucleic acid extract, to compare the sensitivity of rt-RTPCR with conventional RTPCR and conventional nested-RTPCR assays, rt-RTPCR was two to four logs more sensitive than the conventional assays. The rt-RTPCR assay is both simple and rapid with advantages including enhanced sensitivity and a lower risk for cross-contamination making it a useful tool for the detection of rotavirus in various situations including sporadic gastroenteritis, outbreaks, and environmental investigations. G(1) was the predominant type (89%), followed by G(2) (10%), and G(4) (1%). No rotavirus of G(3), G(8), and G(9) types were found. (503).

1.2.3.2.3. **Other techniques**

In their studies of virus inactivation, Pesaro et al (520), assayed infectivity in MA-104 cells after treatments and determined virus replication by the appearance of cytopathic effects and indirect immune peroxidase assays. The indirect immunofluorescent and FC methods proposed by Abad, Pinto and Bosch (2), combine infection of CaCo-2 cells and detection of infected cells by indirect immunofluorescence and flow cytometry (IIF-FC).
The efficiency of this procedure was compared with the standard method of infection of MA-104 cells followed by IIF. The sensitivity was 100 times higher for CaCo-2 than MA-104 cells, respectively, by the IIF-FC method. The ratio of infectious virus particles to total virus particles for a wild-type RV was determined to be $0.5 \times 10^6$ and $0.5 \times 10^4$ for IIF with MA-104 cells and IIF-FC with CaCo-2 cells, respectively, proving that this method was more effective than the classical methods for RV detection. Recently, an integrated cell culture/polymerase chain reaction (ICC/PCR) technique has been developed for the detection of viruses in environmental samples. ICC/PCR allows for detection of infectious viruses in hours compared with days or weeks necessary with cell culture alone. The ICC/PCR approach combines two techniques, conventional cell culture and PCR amplification, using their advantages (detection of infectious viruses and reduced time) and overcoming their limitations (non-cytopatic viruses and detection limit). The technique was able to detect 1 PFU/flask after 20 h incubation instead of 5-7 days required for cell culture (541). An immunochromatographic test for the detection of RV-A also showed high sensitivity (89%) and specificity (99%) compared with ELISA for feces of different species (140).

1.2.4. Epidemiology

1.2.4.1. Incidence and prevalence

Rotaviruses are the leading cause of acute viral gastroenteritis in the young of both avian and mammalian species, including pigs and humans (561, 716). Groups A, B and C (RV-A, RV-B and RV-C, respectively) infect humans and animals, whereas group D, E, F and G (RV-D, RV-E, RV-F and RV-G, respectively) have been found only animals (560,
Group A is responsible for 12% to 71% of diarrhea episodes in developed and developing countries, respectively, (332). Worldwide, RVs cause 138 million cases of illnesses and 600,000 to 870,000 annual deaths. In the U.S, RV accounts for ~2.7 million episodes of diarrhea, 50,000 hospitalizations and 20 deaths per year, so during the first 5 years of life 4 or 5 children will develop a RV diarrhea, 1 of 78 will be hospitalized and 1 in 195,000 will die (648). It is estimated the RV causes more than 3.6 million episode of gastroenteritis, 87,300 hospitalizations, and 200 deaths in children younger than 5 years of age per year in the 25 European Union countries (607). Although RV diarrhea occurs with a high prevalence in developed countries, mortality is low. In developing countries RV causes 130 millions cases and 870,000 deaths per year. So in both developed and developing countries, almost every child under age 5 will develop RV diarrhea, but consequences will be different in developing countries where 1 in 8 will develop severe illness and 1 in 160 will die (212). Adults appear to undergo RV reinfection but with minimal or no clinical manifestations. Rotavirus group B have been associated with several outbreaks of diarrhea in adults in China (632).

The RV-A, RV-B and RV-C infect pigs (203, 351, 559, 560, 716), and both RV-A and non-group A can be detected in the same herd (204, 308, 351). The RV-A are the main agent of viral diarrhea in piglets, accounting for 53% of preweaning and 44% of postweaning diarrhea in swine (20, 179, 200, 561, 716).

1.2.4.2. Transmission and sources

RVs are transmitted by the fecal-oral route, but speculation exists about whether RVs are also transmitted by the respiratory route. The source of infection for young
infants who are not in contact with other infants and young children with gastroenteritis is not clear. Most likely infection is acquired from older children or parents with subclinical infections. Shedding of virus prior and after clinical illness have been documented. Also the environment has been suggested as a possible source because of the persistence of the infection in some newborn nurseries and the high frequency of nosocomial infections in hospitals and the ability of RV to survive on different surfaces. Although RV has been detected in sewage (282, 654, 661), transmission of disease thorough contaminated water is unlikely because high relative humidity results in a rapid loss of infectivity. The RVs display a seasonal pattern of infection in developed countries with epidemic peaks during the winter.

1.2.4.3. Surveillance

Group A, B and C rotaviruses infect pigs and humans (204, 334, 351, 432, 560, 635, 697, 716). Both RV-A and non-group A have been detected in the same herd (20, 179, 200).

The temporal and geographical distribution of human rotavirus G and P types was reviewed by analyzing data collected from 52 countries on five continents published between 1989 and 2004. Four common G types (G1, G2, G3 and G4) in conjunction with P[8] or P[4] represented over 88% of the strains analyzed worldwide. In addition, serotype G9 viruses associated with P[8] or P[6] were shown to have emerged as the fourth globally important G type with the relative frequency of 4.1%. When the global G and/or P type distributions were analyzed in five continents/subcontinents, the P[8]G1 represented over 70% of RV infections in North America, Europe and Australia, but only
about 30% of the infections in South America and Asia, and 23% in Africa. In addition, in Africa (i) the relative frequency of G8 was as high as that of the globally common G3 or G4; (ii) P[6] represented almost one-third of all P types identified; and (iii) 27% of the infections were associated with RV strains bearing unusual combinations such as P[6]G8 or P[4]G8. Furthermore, in South America, uncommon G5 virus appeared to increase its epidemiological importance among children with diarrhea. Such findings have: (i) confirmed the importance of continued active RV strain surveillance; and (ii) provided important considerations for the development and implementation of an effective rotavirus vaccine (566).

In animals group A rotavirus is the main agent of viral diarrhea in piglets, accounting for 53% of preweaning and 44% of postweaning diarrhea in swine (693). One report attributes 89% of all RV diarrhea in commercial pig operations to RV-A (217). Diarrheic animals shed virus in high titer in feces ($10^7$-$10^8$ infectious doses per gram of feces). In pigs, the infection is an age and husbandry-associated disease. Thus RV has been associated with acute diarrhea in pigs weaned at 2-8 weeks of age and during different stages of the suckling period, but not usually during the first 7-10 days of life (56, 465, 689, 700). This difference has been attributed to the level of passively transferred antibodies which remain high during the first week of life and to passive antibodies in colostrum and milk maintained in the gut. The occurrence of RV was significantly higher in 22-28-day-old pigs than in younger pigs during diarrhea outbreaks in 24 farms in Germany (689). Nagy et al (465) reported an 18.6% prevalence of RV-A in postweaning pigs with diarrhea in Hungary. In studies by Janke et al (308), RV-A was detected in 76.4% of nursing pigs and 40.9% of weaned pigs during a diarrhea outbreak.
in a conventional swine operation. Increased numbers of outbreaks or outbreaks in pigs less than 1 week old can occur, if one or more risk factors such as the introduction of a new RV strain, primiparous sows with qualitatively and quantitatively poorer colostrum or poor farm management practices are present on the farm. Farm expansion, early weaning and all-in all-out production were associated with increased numbers of outbreaks in Ontario between 1994 and 1998 (144). A non-previously circulating RV strain, primiparous sows and high population density were considered as the three major risk factors that contributed to an outbreak of diarrhea by RV-A affecting pigs from birth up to 28 days of age (53% of up to 1-week-old pigs, 60% of 8-21-day-old pigs and 52% of more than 21-day-old pigs) in Brazil (32).

The RV-B and RV-C have been identified in humans and pigs. Geyer et al (203) reported RV-B in 4.6% and RV-C in 10.8% by PAGE of samples collected from 1-43-old-day pigs with diarrhea. In the study by Janke et al (308), RV-B and RV-C were detected in 7.4% and 7.5% of nursing pigs and in 18.2% and 22.7% of weaned pigs, respectively, by PAGE. In the U.S. RV-C was identified in fecal samples collected from finishing pigs with diarrhea by IEM, cell-culture immunofluorescence and RT-PCR (351). Group C rotavirus was the cause of enzootic neonatal diarrhea in a swine herd in Quebec reported in 1990. During the outbreaks of diarrhea, the morbidity rate was 100% and case fatality rates were 5 to 10% among 1-2-day-old piglets (461). Group B and C RV were reported as a cause of 12% of 120 outbreaks occurred in Quebec during one year in 2 days to 5 weeks old pigs (427).

Most RV-B or RV-C in humans have been isolated cases, but outbreaks have been reported worldwide (71, 86, 191, 313, 440, 518, 537, 647). However, the finding of a low
prevalence of antibody to RV-C in humans (475, 560, 647) and a higher prevalence in pigs (69, 560, 647) has led to the suggestion that RV-C could be a zoonotic infection of humans (693).

**Co-infections:** A number of authors have reported co-infections of pigs and humans with different RV groups or different G- and P genotypes within RV-A. Janke et al (308) reported co-infection in 8.8% of samples from nursing pigs and in 18.2% of samples from weaned pigs. Co-infection of RV-A and RV-C and RV-C and HuNoV GII was detected in children younger than 3 years of age (523). Mixed infections between G1, G2, G3, G4 and G9 have been described in humans in India and in Europe (178, 307).

### 1.2.5. DISEASE TRANSMISSION

#### 1.2.5.1. The role of the environment

Studies of environmental survival of pathogens under field conditions can be performed only if contamination levels are naturally high, or if the pathogen is introduced into the environment. Pesaro, Sorg and Metzler (520) worked with different non-aerated liquid and semiliquid animal wastes to study *in situ* inactivation of RV. The authors showed that inactivation depended on environmental temperature, pH and type of animal waste. The time required for inactivation was shorter in liquid (pH>8) than in semiliquid (pH<8) wastes. Nevertheless inactivation of RV in swine manure required almost 4 months with a decay rate of less than 1 log_{10} in 6 weeks. In an attempt to estimate the impact of RV contamination on land via cattle slurries, it was estimated that 1m² of land could be contaminated with 2.5 x 10^4 infectious particles, after land application of slurry previously stored for 3 months on an average farm (according to U.K. regulations). The
authors also estimated that in a non-favorable scenario for virus spread, where animal RV will have limited specificity for humans and humans would be partially protected from previous virus exposure, 5000 human infections per year may still arise from contact with calves on dairy farms in the U.K. (125). Similar estimations can be made for swine herds considering that animals are handled by workers during the nursing and weaning time, manure is stored in anaerobic lagoons, and high population density favors disease spread.

Only in exceptional situations, permission will be given to do field studies that require seeding the environment with a pathogen. Therefore a surrogate organism or recombinant particle that models the fate and transport of the pathogenic organism without increasing the risk of disease for the population is desirable. Caballero et al (83) evaluated RV green fluorescent protein-labeled VP2-VP6 recombinant VLPs (GFL-VLPs) as a surrogate for HRV in different environmental scenarios by flow cytometry and antigen capture RTPCR, respectively. Although results did not always have 100% correlation between GFP-VLP and infectious virus, surrogate particles were always equal or more resistant to different treatments. After 1 month in seawater at 20ºC both had the same decay rates; in the presence of 0.2 mg of free chlorine/liter no differences were observed, but the surrogate 2/6 VLPs remained detectable longer than infectious virus when the free chlorine concentration was 1mg/l. The authors also investigated U.V. inactivation in fresh and seawater observing that, again recombinant 2/6 VLP surrogates were less susceptible to U.V. inactivation than infectious viruses.

It has been suggested that RV may be airborne (126). Aerosolized virus can be produced during manure storage, in pig units with forced air ventilation or after pressure cleaning of pens or floors with regular or recycled water. Several authors studied the
survival of RV in the air, and although differences in the results were reported, the general conclusion was that RV can survive in the air long enough to increase the risk of human infection (17). When in the air, RV could be disseminated in the farm and into the nearby population directly or indirectly by air or water. Low relative humidity and rainfall were associated with an increased number of outbreaks among pigs in Venezuela (650). Gratacap-Cavallier et al (224) observed that RV isolated from drinking water in houses of children with recurrent diarrhea due to HRV, were of human and animal origin. Although the RV strains isolated from children and from drinking water differed, the authors suggested that consumption of contaminated water could increase the risk of infection or co-infections. Nevertheless, it is clear that RV detected in drinking water could originate from animal farms. It is also possible that as proposed by Gouvea and Brantly (218) and El-Attar et al (160), different G types may co-exist in the water and a new human reassortant strain may emerge after co-infection of humans with multiple RV strains of animal or human origin.

Most studies have focused on the detection of RV in fresh fecal samples in swine barns. In attempts to evaluate other potential vehicles of transmission, samples of dust, dry feces and effluent were collected from a pig farm and examined for RV-A by ELISA, EM and infectivity of MA-104 cells. The authors found that samples from farrowing and weaning houses were positive by ELISA before and after cell culture, indicating that not only were viral particles present, but also they were infectious. Moreover this study showed that infectious virus was also present in sewage from the farrowing house and in samples collected from a weaning house not used for 3 months
Their study showed that RV can survive for 4 months with a decline rate of 0.5 log10 TCID50 each month, similar to the data presented by Pesaro et al (520).

Overall there are at least four major conclusions to emphasize from these studies. First animal RVs present in the farm are shed in high concentration; second, they have high environmental stability in manure, air, soil and water; and third, they can be disseminated directly or indirectly to other geographical points inside or outside the farm. Finally evidence is accumulating in recent years using newer molecular diagnostic techniques to support the potential zoonotic transmission of RVs. Therefore, efforts should be made to improve animal waste treatments and reduce the risk of RV for disease transmission. Recently, as in the case for other enteric viruses, the effect of environmental technologies on the fate of these pathogens in animal wastes under field conditions is under scrutiny. Previous studies focused on anaerobic inactivation of animal viruses in pits, one of the most commonly used systems at that time (520). As indicated by the authors, at least 4 months were required to inactivate RV, with a potential risk of environmental contamination as a consequence of pit breaks, infiltration into soil or dissemination from the surface. Recently superior environmental technologies were developed to reduce the impact of environmental contamination by different treatments that including high temperature anaerobic digester, biofiltration, solid separation, etc (290).

1.2.5.2. Zoonosis

Zoonoses are infections that under natural conditions are transmitted from animals to humans. The close contact between workers and animals in the farm and the fact that
viruses can survive in the environment and that they can be transmitted to humans directly by air, water and soil or indirectly by food raises concerns for possible interspecies transmission. The presence of RV in livestock is a potential public health problem whose significance is increased by the detection in humans of serotypes and genotypes of animal strains and vice versa. The G3, G4, G5, G11 types and P2B[6], P9[7], P[14], P[19] types are the most common in pigs (75, 143, 219, 285, 334, 561, 716, 718). However human G and P-types such as G1, G2, G9 (36, 115, 535, 567), P[8] and P[6] (535, 567) and bovine G- and P-types such as G6, G8, G10 (219, 501, 528, 535) and, P[1], P[5], and P[11] have also been detected in pigs (220, 432, 528). Human rotavirus strains commonly found in animals have been isolated from children in developed and developing countries. Desselberger, Iturriza-Gomara, and Gray (2001) and Palombo (2002) reviewed these findings indicating that G3 (usually found in cats, dogs, pigs, mice, rabbits and horses), G5 (pigs and horses), G6, G8 and G10 (cattle), and G9 (pigs and lambs) have been isolated from humans worldwide. Moreover, in the last 3 years evidence for the presence of three different porcine strains circulating in humans has been reported. A P[8]G5 strain was reported in children in Cameroon (163), whereas the presence of a G4 strain similar to porcine strains was detected in children in Mexico (375). In 2004, a human strain with all segments (except for VP7) more similar to a porcine strain than to human strains was reported (657), whereas Teodoroff and colleagues (2005) indicated that the most common G9 strain circulating in pigs in Japan was closely related to the G9 strain circulating in humans (635). The emerging G9 strain may have been transmitted to humans from animals, because it has been found in lambs and pigs (180, 567). These uncommon HRVs may have arisen as a whole virus or as a
reassortant between human and animal strains during co-infection of a single cell in animals or humans. Similar to the observations for influenza virus, different RV strains, also with segmented RNA genomes can simultaneously infect one species, rearrange their genomes by exchanging genome segments and emerge as a new strain pathogenic for the original host or for other species.

Controlled experiments have demonstrated that RV circulating in one species can, under the right conditions, emerge as pathogenic reassortants for other species. As an example, rotavirus PP-1 strain (P[7]G3) emerged after passage of a bovine fecal sample in Gn pigs (160). The PP-1 strain was pathogenic for pigs but not for calves. However after the original bovine fecal sample was serially passaged in Gn calves, the emergent strain, CP-1 (P[5]G3), was pathogenic for calves but not for pigs (72). Serial passage in cell culture yielded the reference P[5]G6 bovine strain. These results support the idea that RV exist as a population of reassortants from which a new strain can emerge under appropriate conditions (218). El-Attar and colleagues (2001) proposed that the original bovine fecal sample contained at least P[5] and P[7] and at least G6 and G3 and that the three different strains emerged under different passage/host conditions.

1.3. REFERENCES


134. **Daughenbaugh, K. F., C. S. Fraser, J. W. Hershey, and M. E. Hardy.** 2003. The genome-linked protein VPg of the Norwalk virus binds eIF3, suggesting its role in translation initiation complex recruitment. Embo J **22:**2852-9.


150


161


the contamination of tap water by a small round structured virus. J Hosp Infect 43:149-54.


<table>
<thead>
<tr>
<th>Author</th>
<th>Strains</th>
<th>Segment</th>
<th>Classification</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jiang et al (1997)</td>
<td>H</td>
<td>5</td>
<td>RdRp, Capsid</td>
<td>SaV 2 3 (314)</td>
</tr>
<tr>
<td>Ando et al (2000)</td>
<td>H, Bo</td>
<td>101</td>
<td>RdRp, Capsid</td>
<td>NoV 3 17 (15)</td>
</tr>
<tr>
<td>Smiley et al (2002)</td>
<td>H, Bo, Po</td>
<td>16</td>
<td>RdRp, Capsid</td>
<td>NB-like - - (593)</td>
</tr>
<tr>
<td>Oliver et al (2003)</td>
<td>H, Bo</td>
<td>19</td>
<td>RdRp, Capsid</td>
<td>NoV 3 - (497)</td>
</tr>
<tr>
<td>Wang et al (2005)</td>
<td>H, Po</td>
<td>47</td>
<td>RdRp, Capsid</td>
<td>NoV 3 21 (675)</td>
</tr>
<tr>
<td>Oliver et al (2006)</td>
<td>Bo</td>
<td>17</td>
<td>RdRp, Capsid</td>
<td>BecoV - - (495)</td>
</tr>
</tbody>
</table>

- **a** H: human; Bo: bovine; Po: porcine
- **b** RdRp refers to partial segment and capsid refers to full segment, except where specified.
- **c** First reference about a potentially new genus within the *Caliciviridae* family.
- **d** Second reference about a potentially new genus (*Becovirus* genus, BecoV) within the *Caliciviridae* family.

**Table 1.1:** Most representative contributions to standardize a classification for enteric caliciviruses (ECV)
<table>
<thead>
<tr>
<th>Kit</th>
<th># of samples</th>
<th># of outbreaks</th>
<th>Sensitivity (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Specificity (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Reference technique</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>IDEIA®</td>
<td>479</td>
<td>NA</td>
<td>55.5</td>
<td>98.3</td>
<td>RTPCR</td>
<td>(542)</td>
</tr>
<tr>
<td></td>
<td>244</td>
<td>2</td>
<td>32.7</td>
<td>95.8</td>
<td>RTPCR and/or EM</td>
<td>(534)</td>
</tr>
<tr>
<td></td>
<td>98</td>
<td>35</td>
<td>40.0</td>
<td>NA</td>
<td>RTPCR</td>
<td>(80)</td>
</tr>
<tr>
<td></td>
<td>130</td>
<td>41</td>
<td>62.0</td>
<td>90.0</td>
<td>RTPCR and/or RM</td>
<td>(145)</td>
</tr>
<tr>
<td></td>
<td>158</td>
<td>23</td>
<td>38</td>
<td>96</td>
<td>RTPCR/Southern blot</td>
<td>(136)</td>
</tr>
<tr>
<td></td>
<td>117</td>
<td>0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>76.9</td>
<td>85.9</td>
<td>RTPCR</td>
<td>(692)</td>
</tr>
<tr>
<td></td>
<td>228</td>
<td>0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>60.6</td>
<td>100.0</td>
<td>RTPCR and/or EM</td>
<td>(87)</td>
</tr>
<tr>
<td>RIBASCREEN®</td>
<td>52</td>
<td>NA</td>
<td>35.0</td>
<td>65.0</td>
<td>rt-RTPCR</td>
<td>(569)</td>
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<tr>
<td></td>
<td>130</td>
<td>41</td>
<td>71.0</td>
<td>47</td>
<td>RTPCR and/or EM</td>
<td>(146)</td>
</tr>
<tr>
<td></td>
<td>158</td>
<td>23</td>
<td>36</td>
<td>88</td>
<td>RTPCR/Southern blot</td>
<td>(136)</td>
</tr>
<tr>
<td></td>
<td>92</td>
<td>0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>60.0</td>
<td>97.5</td>
<td>RTPCR</td>
<td>(213)</td>
</tr>
<tr>
<td></td>
<td>117</td>
<td>0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>59.0</td>
<td>73.1</td>
<td>RTPCR</td>
<td>(692)</td>
</tr>
<tr>
<td></td>
<td>228</td>
<td>0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>80.3</td>
<td>100.0</td>
<td>RTPCR and/or EM</td>
<td>(87)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Individual sample  
<sup>b</sup> Only sporadic case samples  
NA: not available

**Table 1.2:** Specificity and sensitivity of commercially available ELISA kits for detection of NoVs in fecal samples from sporadic cases and/or outbreaks of gastroenteritis
<table>
<thead>
<tr>
<th>Sample</th>
<th>Substrate</th>
<th>Organism(^a)</th>
<th>Method</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical</td>
<td>Feces</td>
<td>RV</td>
<td>Dilution, cellulose fiber paper</td>
<td>(690)</td>
</tr>
<tr>
<td>Clinical</td>
<td>Feces</td>
<td>EV</td>
<td>Size-exclusion chromatography, physicochemical extraction</td>
<td>(137)</td>
</tr>
<tr>
<td>Clinical</td>
<td>Feces</td>
<td>NoV</td>
<td>RNAeasy® Mini Kit, Plant and Fungi, QIAamp Viral RNA Mini Kit (QIAGEN)</td>
<td>(572)</td>
</tr>
<tr>
<td>Food</td>
<td>Oysters</td>
<td>PV, NoV, HAV</td>
<td>Organic flocculation, CTAB(^b), PEG(^c) extraction</td>
<td>(23 )</td>
</tr>
<tr>
<td>Food</td>
<td>Oysters</td>
<td>EV</td>
<td>Freon, PEG, chloroform, CTAB</td>
<td>(309)</td>
</tr>
<tr>
<td>Food</td>
<td>Shellfish</td>
<td>NoV</td>
<td>PEG, Freon, ultracentrifugation</td>
<td>(390)</td>
</tr>
<tr>
<td>Food</td>
<td>Oysters</td>
<td>PV, NoV, HAV</td>
<td>Freon, DMSO(^d), glycerol, PEG, Pro-Cipitate</td>
<td>(310)</td>
</tr>
<tr>
<td>Food</td>
<td>Deli ham</td>
<td>NoV</td>
<td>TRIZOL(^e)LS or Tris/glycine buffer, PEG, ultracentrifugation, magnetic beads</td>
<td>(63 )</td>
</tr>
<tr>
<td>Food</td>
<td>Lettuce</td>
<td>CaCV, EV, NoV</td>
<td>Compare 3 elution, 3 concentration, and 3 RNA extraction methods</td>
<td>(386)</td>
</tr>
<tr>
<td>Food</td>
<td>Raspberries</td>
<td>NoV</td>
<td>Elution, centrifugation, CAT-floc</td>
<td>(556)</td>
</tr>
<tr>
<td>Food</td>
<td>Cream, lettuce</td>
<td>CaCV</td>
<td>Compare 3 elution technique and 2 commercial kit for RNA extraction</td>
<td>(553)</td>
</tr>
<tr>
<td>Food</td>
<td>Oysters</td>
<td>HAV, NoV</td>
<td>Elution, PEG, centrifugation, Tr(_i) (SIGMA)/chloroform, Oligo(dT)-beads</td>
<td>(352)</td>
</tr>
<tr>
<td>Food</td>
<td>Berries, lettuce</td>
<td>PV, HAV, NoV</td>
<td>Tris-Glycine or Beef extract elution buffer, PEG, organic extraction</td>
<td>(154)</td>
</tr>
<tr>
<td>Food</td>
<td>Oysters</td>
<td>NoV</td>
<td>Several elution buffers, PEG, Proteinase K, phenol/chloroform, silica gel</td>
<td>(76 )</td>
</tr>
<tr>
<td>Food</td>
<td>Several food</td>
<td>NoV</td>
<td>Magnetic beads</td>
<td>(358)</td>
</tr>
<tr>
<td>Food</td>
<td>Oysters</td>
<td>NoV</td>
<td>Magnetic beads</td>
<td>(415)</td>
</tr>
<tr>
<td>Food</td>
<td>Oysters</td>
<td>NoV</td>
<td>Comparison of 4 methods</td>
<td>(572)</td>
</tr>
<tr>
<td>Environment</td>
<td>Water</td>
<td>PV, HAV, NoV</td>
<td>Zirconium hydroxide precipitation</td>
<td>(130)</td>
</tr>
<tr>
<td>Environment</td>
<td>Sludge, soil</td>
<td>EV</td>
<td>Beef extract, size-exclusion chromatography</td>
<td>(616)</td>
</tr>
<tr>
<td>Environment</td>
<td>Soil</td>
<td>EV</td>
<td>Physical separation, solvent extraction, size/ion-exchange chromatography</td>
<td>(617)</td>
</tr>
<tr>
<td>Environment</td>
<td>Water</td>
<td>NoV</td>
<td>Membrane filtrate</td>
<td>(706)</td>
</tr>
<tr>
<td>Environment</td>
<td>Water</td>
<td>PV</td>
<td>Commercial kits: magnetic beads with/out centrifugation, silica gel columns</td>
<td>(74 )</td>
</tr>
</tbody>
</table>

\(^a\) RV: Rotavirus; EV: enterovirus; NoV: Norovirus; SaV: Sapovirus; PV: Poliovirus 1; HAV: Hepatitis A; CaCV: canine calicivirus

\(^b\) Cetyltrimethyl ammonium bromide; \(^c\) Polyethylene glycol; \(^d\) Dimethyl sulfoxide

**Table 1.3:** Techniques for virus recovery from food, environmental and clinical samples
Figure 1.1: Immune electron microscopy of two members of the *Caliciviridae* family: Members of *Sapovirus* genus present a characteristic “Start of David” appearance for which caliciviruses were named (a); however other members were originally named as Small Round Structured Viruses (currently Noroviruses) because of their indistinct “feathery” outer edge (b). Bar= 50 nm

[From http://www.virology.net/Big_Virology/BVRNACalici.html by F.P. Williams]
Figure 1.2: Phylogenetic analysis of complete capsid aa sequences of 141 NoV strains. Five genogroups (G) and 29 clusters (# in boxes) were described [Zheng, D.P., Ando, T., Fankhauser, R.L., Beard, R.S., Glass, R.I. and Monroe, S.S. (2006). Virology, 346; 312-323]
Figure 1.3: Phylogenetic analysis of complete capsid aa sequences of 39 SaVs strains. A total of 5 genogroups (roman numbers) and 13 genotypes (arabic numbers) were described [Okada, M., Yamashita, Y., Oseto, m., Ogawa, T., Kaiho, I., and Shinozaki, K. (2006). Virus Genes 33:157-161]
Figure 1.4: Schematic representation of the genome organization in open reading frames (ORF) of a) NoV (Norwalk, Genbank accession number M87661); b) Vesivirus (FCV, M86379); c) SaV (Manchester, X86560); d) Lagovirus (RHDV, M67473); e) Becovirus or Nabovirus (Nebraska, NC_004064). N-term: N-terminal protein; 3CLPro: Protease, 3DRdRp: RNA- dependent-RNA-polymerase; VP1: capsid; VP2: small basic protein. Numbers indicate nucleotide positions.
**Figure 1.5:** Norwalk virus-like particle (NV-VLP) structure was solved by cryo-electron microscopy and X-ray crystallography (top, surface representation; bottom, cross section). Each monomeric capsid protein (right) is divided into a N terminal arm facing the interior of the VLP (green), a shell domain (S-domain; yellow) and the protruding domain (P-domain). The P-domain is divided into subdomain P1 (red) and P2 (blue) at the most distal surface. [Hutson, A.M., Atmar, R.L. and Estes, M.K. (2004). Trends in Microbiology, 12 (6); 279-287]
**Figure 1.6:** Replication scheme of ssRNA+ virus. Schematic representation of a potential replication cycle for ECV (1) Translation of genomic RNA into non-structural polyprotein precursor; (2) Proteolytic cleavage of polyprotein precursor; (3) Synthesis of anti-genomic or anti-subgenomic RNAs catalyzed by 3D<sup>RdRp</sup>; (4) RNA transcription into subgenomic RNAs catalyzed by 3D<sup>RdRp</sup>; (5) RNA replication; (6) Translation of subgenomic RNA encoding structural proteins; (7) Virus assembly
Figure 1.9: The biosynthesis pathways of the ABH and Lewis HBGAs on the type 1 precursor. Enzyme A: N-acetyl-galactosamine-transferase; enzyme B: galactosyltransferase; Fuc: L-fucose; FUT2: \(\alpha(1,2)\) fucosyltransferase; FUT3: \(\alpha(1,3\text{ or }1,4)\) fucosyltransferase; Gal: D-galactose; GalNAc: N-acetyl-galactosamine; GlaNac: N-acetyl-glucosamine. [Tan, M., and Xi Jiang (2005) Trends in Microbiol 13 (6): 285-293]
Figure 1.10: Rotavirus structure: The complete virion (triple layered) contains the 11 segments of the dsRNA genome. The polyacrylamide gel on the left shows the 11 segment and the encoded proteins (central column). On the right, cut-away of the triple layered virus particle showing: VP2 layer in green, VP6 layer in blue, VP7 layer in yellow, VP4 spikes in red. The flower-shaped VP1-VP3 transcription complex is attached to the inside of the VP2 layer. [Adapted from Pesavento et al (2006) Curr Top Microbiol Immunol 309: 189-219.]
CHAPTER 2

HUMAN AND ANIMAL ENTERIC CALICIVIRUSES IN OYSTERS FROM DIFFERENT COASTAL REGIONS OF THE US.

2.1 SUMMARY

Foodborne diseases are a major cause of morbidity and hospitalizations worldwide. Enteric caliciviruses are capable of persisting in the environment and in the tissues of shellfish. Human noroviruses (HuNoVs) have been implicated in outbreaks linked to shellfish consumption. The genetic and antigenic relatedness between human and animal enteric caliciviruses suggests a possibility for interspecies transmission. To determine the occurrence of human and animal enteric caliciviruses in U.S. market oysters, we surveyed regional markets. Oysters were collected from 45 different bays along the US coast during summer and winter of 2002-2003. Samples were analyzed by RTPCR and results were confirmed by hybridization and sequence analysis. Nine samples (20%) were positive for HuNoVs GII after hybridization. Animal enteric caliciviruses were detected in 10 samples (22%). Seven of these were positive for porcine norovirus GII and one sample was positive for porcine sapovirus after hybridization and confirmation by sequencing. Bovine noroviruses were detected in two samples and
confirmed by sequencing. Five HuNoV samples sequenced in the polymerase region were similar to NoVs GII US 95/96 subset (GII-4) previously implicated in diarrhea outbreaks. Different seasonal and state distributions were detected. The presence of animal enteric caliciviruses was associated with states with high livestock production. Although the presence of human caliciviruses in raw oysters represents a potential risk for gastroenteritis, disease confirmation by outbreak investigation is required. The simultaneous detection of human and animal enteric caliciviruses raises concerns for human infection or co-infection with human and animal strains that could result in genomic recombination and emergence of new strains.

### 2.2 INTRODUCTION

Foodborne diseases are a major cause of morbidity and hospitalization worldwide. Each year 76 million illnesses, more than 300,000 hospitalizations and 5,000 deaths are reported in the US alone, indicating that 1 in 4 Americans develop foodborne illness each year and at least 1 in 1000 is hospitalized (40, 41). Despite major advances and improvements in food and water quality (32), diagnostic methods (3, 56) and surveillance systems (8, 40), foodborne diseases remain a global public health problem. Only 20% of the foodborne illnesses are attributed to known pathogens. Although attention has focused on preventing bacterial infections, viral agents are estimated to account for more than two-thirds of the foodborne illnesses caused by known pathogens (41), but no methods are currently available (antivirals, vaccines) for their prevention or treatment.

The percent of the outbreaks associated with seafood is around 10-20% in countries such as US or Australia, but this percentage increases to 70% in countries with
higher seafood consumption such as Japan or wherever seafood is eaten raw (7, 36). Seafood includes both fish and shellfish (molluscs and oysters). Molluscs are filter feeders that can concentrate in their tissue particles present in the surrounding water by more than four-fold, including fecal coliforms and viruses (6). An etiological agent is confirmed in only 44% of the outbreaks associated with seafood and 47% of the outbreaks associated with seafood and with confirmed etiology are caused by viruses (7, 64).

Viruses in the family Caliciviridae are divided into 4 genera: Norovirus, Sapovirus, Vesivirus and Lagovirus. Human and animal caliciviruses associated with gastroenteritis belong to the Norovirus and Sapovirus genera. Human Noroviruses (HuNoVs) cause illness in people of all ages, whereas Human Sapoviruses (HuSaVs) cause illness primarily in children (19). The Norovirus genus is divided into 5 genogroups (GI, GII, GIII, GIV and GV) that can be subdivided into 8, 19, 2, 1 and 1 genotypes, respectively based on phylogenetic tree topology and distance analysis of the capsid gene (62). Human noroviruses belong to GI, GII and GIV. The Sapovirus genus is also divided in 5 genogroups and subdivided in 3, 3, 1, 1, and 1 genotypes, respectively, based on similar analysis of the capsid sequence (14). Human sapoviruses belong to GI, GII, GIV and GV.

Animal enteric caliciviruses cause gastroenteritis in calves and pigs and have also been isolated from healthy pigs. Porcine Norovirus (PoNoV) was detected in Japan and Europe and recently in the US (55, 57, 62). The PoNoVs detected in Japan and the US belong to 3 different genotypes within GII, the most widely detected norovirus genogroup in humans. Bovine noroviruses (BoNoVs) have been detected in Germany and England and in the US and they belong exclusively to GIII (9, 39, 53). Porcine sapovirus (PoSaV) has
emerged as an important pathogen associated with diarrhea and subclinical infections among pigs of all ages, since it was discovered in 1980 (15, 21).

Low infectious doses (19), prolonged asymptomatic shedding (17), environmental stability (49) and great strain diversity (1, 14) increase the risk of infections by this virus family. Moreover, the identification of closely related animal enteric caliciviruses in cattle and pigs, and the existence of recombinants within BoNoVs (23, 46), PoNoVs, HuNoVs and also HuSaVs (26, 29, 30) raises concerns for possible human infections or co-infection of animals or humans with human and animal enteric caliciviruses.

In the US, HuNoVs accounted for 93-96% of the outbreaks of non-bacterial acute gastroenteritis submitted to CDC from 1997 to 2000 (13). It is estimated that 50-66% of all foodborne illness of known etiology is due to HuNoVs, and 52% of gastroenteritis associated with consumption of raw or partially cooked shellfish are attributable to HuNoVs, placing them as leading cause of seafood-associated (especially oysters) foodborne illness (7). The first oyster and NoV-associated gastroenteritis outbreak involved about 2000 persons and it was reported during the summer of 1978 in Australia (43). Since then, several reports worldwide have identified NoVs-contaminated oysters in association with outbreaks of gastroenteritis (4, 11-13, 31, 35, 50, 51).

The objectives of this study were to conduct a survey of regional markets in the US to determine the presence of human and animal enteric caliciviruses in market oysters and to compare the calicivirus strains detected from these market oysters with strains previously detected and associated with human gastroenteritis.
2.3 MATERIALS AND METHODS

**Shellfish sampling:** Oysters were collected from bays on the East, West and Gulf coasts from which licensed shippers (Interstate Shellfish Sanitation Conference Shippers List) harvest oysters for consumer markets. When identified, each bay was assigned a number and placed into an Excel spreadsheet, randomized, and 12 bays were chosen per coast. Twelve bays in the West coast, 12 in the East coast and 9 in the Gulf coast corresponding to 4, 6 and 3 states on each coast, respectively, were sampled during the summer of 2002 (May 2002- September 2002). During winter (November, 2002- March, 2003), 4 bays in the West coast, 4 in the East coast and 4 in the Gulf coast were sampled again for a total of 610 oysters (at least 12 from each bay on each season) that belonged to 45 bays (Table 2.1). A lower harvesting rate during winter was the reason for the reduced sampling during this season. Oysters were purchased from farmers, wholesalers and retailers and at least 12 oysters were obtained from each bay for this study. A specific code according to coast was assigned to each bay: West coast, 1- to 12W; East coast, 1- to 12E; and Gulf coast, 1- to 9G. Each bay was considered one sample and the oysters from each bay collection were pooled. Oysters were kept at -20 ºC and shipped overnight to our lab.

**Shellfish processing:** For each bay, oyster samples were rinsed in water prior to opening, and counted. Oysters were shucked with a sterile knife and the oyster tissue was removed. The digestive diverticula was removed and dissected with sterile scissors and forceps. Digestive tissues from oysters with the same collection date and bay were pooled, homogenized and homogenates were then subdivided into aliquots of 1.5g. Homogenates were frozen (-20º C) until analysis. Number of oysters from each bay,
harvest and arrival date, tissue weight (entire oyster tissue without shell), and digestive tissues weight were recorded.

**Virus elution and concentration:** Three different protocols were assessed to determine virus recovery and to remove natural PCR inhibitors present in the samples. Protocols for virus elution, concentration and extraction of nucleic acid (NA) from oysters were standardized by using oysters artificially seeded with human or animal enteric caliciviruses available in our laboratory. Three different protocols (Protocol 1, 2 and 3, Fig 2.1) were tested. Briefly, different samples were tested for human and animal enteric caliciviruses with broadly reactive and specific primers (Table 2.2). Three of them, confirmed negative, were artificially contaminated with 3 different inocula: A) HuNoV; B) PoSaV or C) BoNoV positive fecal samples in high (AH, BH, CH, respectively) and low (AL, BL, CL, respectively) concentrations (Table 2.3). The six batches were processed by protocols 1, 2 and 3 and the presence of virus was tested by RTPCR with specific primers for each virus strain as described below. The presence of natural PCR inhibitors was tested by using an internal control (IC) and RTPCR with primers NV/35 and NV/36, specific for this IC as described below.

Natural PCR inhibitors were detected in batches CH and CL by Protocol 1, in all of them by Protocol 2 and in batches BH, BL, CH and CL by Protocol 3, suggesting that Protocol 1 is the most effective to remove PCR inhibitors among the protocols tested. Samples with inhibitors were diluted 1:10 in DEPC-treated water to overcome inhibition, and RTPCRs with specific primers for each strain were performed. Batches AH and AL were tested with primers HS40F/R, batches BH and BL were tested with primers PEC-65/66 and batches CH and CL with primers CBECU-F/R. RNAs were diluted 1:10,
1:100, 1:1000 and 1:10000 to determine recovery. Several non-specific RTPCR products were detected in all of the groups but specific amplification of the target was detected only in batches AH, AL, BH, and CH using Protocol 1. However RNA titers recovered were lower than the titer of RNA in the inocula used.

In summary, Protocol 1 was more efficient than the others to remove PCR inhibitors and to recover viral nucleic acid from oysters, although the amount of RNA detected was lower than that originally inoculated. Briefly for Protocol 1, an aliquot of 1.5 g of homogenate was thawed on ice and processed as previously described by Atmar et al 1995 (3) with minor modifications. The homogenate was homogenized in PBS (pH 7.4) with a grinder and transferred to a centrifuge tube. Chloroform-butanol was added to remove tissues and Cat-Floc T (20% polydiallyldimethyl ammonium chloride) (Calgon Corp.) was added to coagulate proteins. After centrifugation the supernatant was recovered and PEG 6000 was added to concentrate the virus. After centrifugation, the pellet was resuspended in 3 ml of DEPC-treated water for nucleic acid extraction.

**Nucleic acid extraction:** Nucleic acids were purified from concentrated virus as previously described by Atmar et al (3) with minor modifications. Briefly, after digestion of the pellet with Proteinase K (20mg/ml) (AMRESCO), double extraction was performed with 70% Phenol-Chloroform-water (Applied Biosystems) and Chloroform/Isoamylic alcohol (24:1). The aqueous phase was precipitated with ethanol, the pellet resuspended in DEPC-treated water, and 5% cetyltrimethylammonium bromide (CTAB, Sigma) was added to remove PCR inhibitors. After 15 min incubation at room temperature, samples were centrifuged 30 min at 14,000g and the supernatant was discarded. The pellet was resuspended in 1M NaCl and reprecipitated with ethanol and
3M sodium acetate (pH 5.2) for 30 min at -80ºC. The precipitated nucleic acids were resuspended in 50 µl of DEPC-treated water with 0.8 U/ul RNasin (Promega, Madison, WI, USA). The presence of natural PCR inhibitors was tested by using an internal control (IC) and RTPCR with primers NV/35 and NV/36, specific for this IC as described below.

**Detection of PCR and RTPCR inhibitors in oysters:** An internal control (IC) was used in RTPCR to assess the presence of RTPCR inhibitors. The IC (kindly provided by Drs Estes and Atmar), was generated from a DNA clone containing the region amplified by primers NVp35 and NVp36 (4487-4956 bp) with a 123 bp-deletion that yielded, after RNA synthesis by SP6 polymerase, an RNA amplicon of 347 bp (50). One microliter of IC was added to each sample during the RTPCR and primers NVp35/NVp36, specific for this IC, were used (Table 2.2). Briefly, for RT, 3 µl of RNA added to 22 µl of RT mixture containing 2.5 µl of 10X PCR Buffer (100mM Tris-HCl [pH9.0 at 25ºC], 500mM KCl and 1% Triton® X-100), 2.5 µl of 25 mM MgCl₂, 1 µl of 10 mM dNTPs, 1 µl of 50uM reverse primer NVp35, 2 U of Avian myeloblastosis virus reverse transcriptase (AMV-RT, Promega) and 8 U of RNasin (Promega) were incubated at 42º C for 1 h, followed by heat inactivation at 94º C for 3 min. For PCR, another 25 µl PCR mixture containing 2.5 µl of 10X PCR Buffer, 2.5 µl of 25 mM MgCl₂, 1 U of Taq DNA polymerase and 1 ul of 50 µM forward primer NVp36, was added to the RT reaction. After initial denaturation at 94º C for 3 min, 40 cycles of 94ºC for 30 sec, 50º C for 30 sec, 72º C for 30 sec were performed follow by a final extension of 72ºC for 10 min. Amplified products were analyzed by 9% acrylamide gel electrophoresis, stained with ethidium bromide and visualized under U.V. light. If inhibition was detected, the RNA template was diluted 1:10 to overcome it and the RTPCR was repeated.
**RTPCR for calicivirus detection:** Several primer sets combined into nine different RTPCR protocols and 1 seminested-PCR were used to detect human and animal enteric caliciviruses. Based on the conserved RdRp region, broadly reactive primers for both NoVs and SaVs, and specific primers for human or animal NoVs or SaVs have been used as described in Table 2.2. The RTPCRs with broadly reactive primers NVp110/p290 or HuNoV genogroup specific primers NI (GI), NV-4611 (GII) and NVp36 (GI), were performed in two steps. For RT, 4 µl of reverse primer NVp110 (50µM) were added to 12 µl of RNA and were incubated at 94 ºC during 2 min and transferred to ice. Twenty four microliters of RT mixture containing 11 µl of DEPC-treated water, 4 µl of 10X PCR Buffer (100mM Tris-HCl [pH: 9.0 at 25º], 500 mM KCl, and 1% Triton® X-100) (Promega), 4 µl of 25 mM MgCl₂ (Promega), 4 µl of 10 mM dNTPs, 5U of AMV-RT (Promega) and 20 U of RNasin were added and incubated at 42º C for 1 h, followed by heat inactivation at 94º C for 3 min. For PCR, 10 µl aliquots of the RT product were transferred to 4 different PCR tubes and 40 µl of PCR mixture containing 30.8 µl of DEPC-treated water, 4 µl of 10X PCR Buffer, 4 µl of 25 mM MgCl₂, 5 U of Taq DNA polymerase and 1 µl of 50 uM of one forward primer (p290, NI, NV4611 or NV36) were added to each tube. After initial denaturation at 94º C for 3 min, 40 cycles of 94º C for 30 sec, 50º C for 30 sec, 72º C for 30 sec were performed follow by a final extension of 72ºC for 10 min.

RTPCR with primers JV12Y/13I was performed also in two steps as previously described by Vennema et al, with minor modifications (58). For RT, 4 µl of reverse primer JV13I (50uM) were added to 5 µl of RNA and were incubated at 94ºC during 2 min and transferred to ice. Six microliters of RT mixture containing 2.3 µl of DEPC-treated water, 1.5 µl of 10X PCR Buffer, 1.5 µl of 25 mM MgCl₂, 0.3 µl of 10 mM dNTPs, 2 U of AMV-
RT and 8 U of RNasin were added and incubated at 42º C for 1 h, followed by heat inactivation at 94º C for 3 min. For PCR, 5 µl aliquote of the RT product were added to 45 µl of PCR mixture and PCR was performed as previously described (58).

For animal enteric caliciviruses, RTPCR was performed with primer set CBECU-F/CBECU-R for BoNoVs as previously described by Smiley et al (53), with primer set PEC66-Bio/65 for PoSaVs as previously described by Guo et al (21) and for PoNoVs, RTPCR was performed with primer set PNV7-Bio/8 as described by Wang et al (62).

Semi-nested PCR was performed, using the product of NVp110-NV4611 RTPCR as template for primers NVp110-NI. Briefly, 3 µl of RTPCR product were added to 47 µl of PCR mixture containing 5 µl of 10X PCR Buffer, 5 µl of 25 mM MgCl₂, 1 µl of 10 mM dNTPs, 1 µl of 50uM forward primer NI, 1 µl of 50uM reverse primer p110, and 1 U of Taq DNA polymerase. After initial denaturation at 94º C for 3 min, 40 cycles of 94ºC for 30 sec, 50º C for 30 sec, 72º C for 30 sec were performed follow by a final extension of 72ºC for 10 min. All amplified products were analyzed by electrophoresis using 9% acrylamide gels, staining with ethidium bromide and visualized under U.V.

**Hybridization assay for HuNoVs:** Dot blot analysis was applied to confirm RTPCR products as previously described by Le Guyader et al (34). Probes GGIα, GGIβ and GGI (59) (Table 2.2) were labeled with digoxigenin using the 3’DIG Oligonucleotide tailing kit (Boehringer Mannheim). The RTPCR products (NVp110/p290 or JV12Y/13I) were diluted in buffer (10mM Tris-HCl [pH 8.0], 1 mM EDTA [pH 8.0]) and denatured at 95ºC. The RTPCR products were blotted onto a positively charged nylon membrane (Sigma) under vacuum and fixed by UV. Positive controls were introduced on each membrane. After prehybridization at 50ºC for 30 min,
hybridization was performed for 2 hs at 50°C. Hybridized probes were detected by chemiluminescence using CDP-Star-ready to use kit (Roche) according to the manufacturer’s protocol.

Microwell probe capture hybridization assay for PoNoV and PoSaV: For PoNoV and PoSaV a microplate probe capture hybridization assay with specific probes was applied to confirm RTPCR products as previously described by Wang et al (61). Briefly, EIA/RIA 8-wells strips (Corning Inc, Corning, NY) were coated with 100ng/well of probes PoNoro1A/1B/1C or PoSapo1A/1B/1C to detect PoNoVs or PoSaVs, respectively. The RTPCR products (PNV7-Bio/8 or PEC66-Bio/65) were diluted 1:1 in denaturation buffer and transferred to the strips. Positive and negative RTPCR controls were included. After incubation, hybridized products were detected with Neutravidin™-Horseradish Peroxidase conjugate (Pierce Biotechnology, Rockford, IL) and tetramethylbenzidine (TMB) as substrate (KPL, Gaithersburg, MD). The absorbance was read by a spectrophotometer at 450 nm.

Sequence analysis: RTPCR products were purified from polyacrylamide gel by Gebaflex kit as described by manufacture (Bioworld, OH). The DNA was sequenced directly (when the amount of DNA was enough) or after cloning into pCR2.1-TOPO (T/A) vector (Invitrogen), using BigDye Terminator Cycle chemistry and 3700 DNA Analyzer (Applied Biosystems, Foster, CA). Sequence data were aligned using Lasergene software package (DNASTAR Inc., Madison, WI, USA) and compared with the published sequence using Basic Local Aligment Search Tool (BLAST) and Clustal methods.
2.4 RESULTS

In this study, a total of 610 oysters that represent 45 bays (or 45 samples) were purchased from different markets around the U.S. coasts (Table 2.1). All of them were supplied by licensed shippers that harvest oysters from bays on the East, West and Gulf coasts approved for human consumption. To assess the presence of human or animal enteric caliciviruses, 33 bays (12 in the East, 12 in the West and 9 in the Gulf coasts) during summer and 12 bays (4 in each coast) during winter (a total of 45 bays) were randomly selected. Oysters harvested from one bay and at one time were considered one sample. Each sample was composed of at least 12 oysters. Tags were checked to determine the harvest location.

Crude shellfish extracts often contain low virus concentrations compared with feces, in addition to natural RTPCR inhibitors. To overcome these problems, virus was eluted from the oyster tissue and concentrated. In addition, CTAB was included in the extraction of RNA to remove inhibitors and the presence of inhibitors after RNA extraction was assessed by introducing an IC. The use of the IC showed that 32 of 45 samples (71%) displayed inhibition (data not shown). To overcome this problem, RNAs were diluted 1:10 in DEPC-treated water before analysis.

Primarily HuNoVs GII were detected in oysters as determined by RTPCR and hybridization: The HuNoVs were detected by one or two genogroup specific primer sets in 44% (20/45) of the samples (Table 2.4). In addition HuCV were detected with broadly reactive primers, but not NoV specific primers in 3/45 (7%) samples. No positive samples were detected with primer set NVp110-NVp36 for HuNoVs GI.
After hybridization, 9 of 45 RTPCR products with primer set NVp110-p290 or JV13I/12Y were positive with probe GGII, confirmed as HuNoVs GII. These 9 samples were also positive with the GII specific primer pair (Table 2.4). These samples correspond to West Coast (3 in Oregon, 1 in Alaska, 1 in Washington), East Coast (2 in New York) and Gulf coast (1 in Mississippi and 1 in Louisiana). No positives were detected with GGlα and GGlβ which agrees with the RTPCR results in which no positives were detected with NoVs GI specific primer set (NVp110-NVp36).

**Animal enteric caliciviruses were detected in oysters mainly from the West Coast as determined by RTPCR or hybridization:** Fifteen of 45 samples (33%) were positive with specific primer sets for animal enteric caliciviruses (Table 2.5); 2 of these were positive with BoNoV specific primer set, 1 was positive with the PoSaV specific primer set and 12 with the PoNoV primer set. Microwell probe capture hybridization assay was applied to confirm RTPCR results for PoNoV and PoSaV. Seven of 12 samples positive for PoNoV by RTPCR were positive by hybridization and confirmed as PoNoV within GII. Six of these 7 samples (86%) corresponded to the West Coast (3 in Oregon, 1 in California, 2 in Washington), and 1 sample to the East Coast (Delaware). In the case of PoSaV, the RTPCR product amplified with primer set PEC66-Bio/65 was also confirmed by hybridization as PoSaV within GIII. The only positive sample for PoSaV was from Oregon. Of the two samples positive with primers for BoNoV, one was from Washington and the other from Oregon (Table 2.5).

**The HuNoVs detected in oysters belong to NoVs GII-4 as determined by sequence analysis.** Among the 9 samples positive for HuNoVs by RTPCR and hybridization, 5 sequences were obtained from amplicons amplified with NVp110/NI
These samples were from Washington (3W), Oregon (1W), Louisiana (12G), Mississippi (10G) and New York (5E). Norovirus strains detected in oysters from these states belonged to GII-4 and they were closely related to the 95/96-U.S. subset detected in diarrhea outbreaks in the U.S and 7 other countries during the same period. The 7 amplicons amplified with specific primers for PoNoVs (PNV7-Bio/8) were also sequenced. All of them were confirmed as NoVs GII and originated from bays in Washington (3W, 6W), California (7W), Oregon (1W, 2W, 10W) and Delaware (8E) (Table 2.6). The two amplicons amplified with specific primers for BoNoVs were also sequenced and confirmed as NoVs GIII.

Human Noroviruses were detected among the 3 coasts, whereas animal enteric caliciviruses were mainly detected in the West coast: Samples were collected from a total of 13 different states. Results were organized by season, coast, state and bay (Table 2.6, Fig 2.2). In summer, 33 bays from 13 states were sampled, whereas in winter, 12 of these bays from 7 states were sampled again. Human and/or animal enteric caliciviruses were detected at least one time in one bay in 11 of 13 states (23/45 bays) by RTPCR or RTPCR and hybridization, with negative results in NJ and SC (Table 2.6).

Human Noroviruses were detected in 10 states (WA, OR, AK, ME, VA, NY, DE, FL, LA and MS) with negative results in NJ, CA and SC. Although it was clear that when HuNoV was detected in a state, it was present in both seasons (WA, NY and ME), only in summer (OR) or only in winter (VA, FL), it was also clear that within each state, some bays were positive and others were negative at different times (Table 2.6). In LA, samples from 3 bays (6G, 7G and 12G) tested positive for HuCVs, whereas the other 2 bays (5G and 11G) tested negative. Four of 6 bays were negative in FL. In NY, 3 of 4
bays were positive (4E, 5E and 11E). In the case of AK and WA, 4 bays were sampled from each state. In both states, 2 bays were negative but HuNoVs were detected in the other 2 (3W, 4W, 8W and 11W). All samples from OR were positive for HuNoVs (1W, 2W and 10W).

Animal enteric caliciviruses were detected in 3 of 4 West coast states sampled (OR, WA, and CA) and in only one East coast state (DE) (Table 2.6, Fig 2.2). In OR, PoNoV was detected in the 3 bays studied, PoSaV in one (1W) and BoNoV in one (2W). Two of 4 bays were positive for PoNoVs in WA (3W and 6W), whereas BoNoV was detected in a third bay (4W) and one bay was negative for all of them. One bay was sampled in CA and it tested positive for PoNoV, as well as one of two bays sampled in DE.

**Human and animal enteric caliciviruses were detected in both seasons in the same or different bays:** Human caliciviruses were detected in both seasons by RTPCR with different primers sets, but only samples from summer were also positive by hybridization with probe GGII (HuNoV GII) (3). Human Noroviruses were detected only in summer in OR (1W, 2W and 10W) and only in winter in VA and FL (3E and 1G). However in some states such as WA, NY and ME HuNoVs were detected in both seasons, in same or different bays. During summer in FL, samples from one bay (8G) tested positive for HuCV, whereas the other 2 bays tested negative, including oysters from bay 1G that were later positive for HuNoV in winter. Other 3 samples collected during winter in FL were negative. In NY, 3 of 4 bays were positive during summer (4E, 5E and 11E) and the bay that was sampled in both seasons was also positive in winter (4E). In WA, 4 bays were sampled during summer resulting in 2 positive and 2 negative bays for HuNoVs. However, the 2 positive bays were also sampled in winter, but only
oysters from bay 3W were positive for HuNoVs. In OR, HuNoV GII was detected in all bays sampled during summer. But in winter, samples collected in 2 of these bays (1W and 2W) were negative with all the human calicivirus primers used (including GII specific primers that showed a positive reaction with summer samples from these bays).

Animal enteric caliciviruses were detected in both seasons. However whereas those 7 samples positive to PoNoV were from OR, WA, CA and DE bays sampled during summer, the 2 samples positive to BoNoVs and one positive to PoSaVs were detected only in 2 (2W and 4W) and 1 bay (1W), respectively during winter in OR and WA.

**Human and animal enteric calicivirus were simultaneously detected in oysters collected from the same bay during same season:** In the states surveyed with high cattle or swine livestock production such as OR and WA, HuNoVs and different animal enteric caliciviruses were detected in the same bay during the same or different seasons (Table 2.6, Fig2.2). In OR, HuNoVs and PoNoVs were simultaneously detected in oysters collected from all bays sampled during summer (1W, 2W and 11W), whereas in winter PoSaVs and BoNoVs were detected in different bays (1W, 2W), but no PoNoVs or HuNoVs were detected. In WA, HuNoVs were detected in one bay (4W), PoNoVs in another bay (6W) and both were detected in bay 3W during summer. In winter only HuNoVs were detected in bay 3W, and BoNoV, but not HuNoV, was detected in bay 4W.

**2.5 DISCUSSION**

Human health problems associated with bivalve shellfish are well-documented (32). Viruses are strict intracellular pathogens that can not replicate in food or water. Therefore, foodborne viral infectious diseases will depend on initial concentrations of
virus in the food, host susceptibility, virus stability, and the dose required for infection. Although SaVs preferentially cause disease in children, NoV infections do not distinguish between children, the young or adults, and host susceptibility is based on ABH-histoblood group antigens with different patterns of attachment for each strain (25). In addition, NoVs and SaVs are non-enveloped viruses, resistant to disinfection, heat and pH changes and the infectious dose is as low as 10-100 particles (42). Therefore enteric caliciviruses (particularly NoVs) are ideally suited to their role as foodborne disease associated pathogens explaining why an estimated 50-66% of all foodborne illnesses of known etiology are caused by HuNoVs (64).

Most foodborne disease outbreaks are due to direct contamination of food or water by a food handler during food distribution at the end of the chain, but may also occur at any point from harvest to table. In the case of seafood, the association of shellfish-transmitted infectious diseases with sewage pollution, symptomatic or asymptomatic cases or illegal overboard sewage discharges into harvest areas are well established worldwide, and human enteric viruses are the most common etiological agent transmitted by bivalve shellfish (48). In U.S, the first seafood-associated NoV outbreaks occurred in 1980 due to oysters harvested in Florida and contaminated with NoVs (20). Since then several outbreaks have been traced back to the harvest location in the same or different states (4, 11). Oyster-related outbreaks will continue unless frequent monitoring and more stringent control measures are established.

In this study, oysters were purchased from different markets around the coastal US from oyster beds, approved for human consumption and supplied by licensed shippers that harvest oysters from bays on the East, West and Gulf coasts. To assess the presence
of human or animal enteric caliciviruses, 33 bays (12 in the East, 12 in the West and 9 in the Gulf coasts) during summer and 12 bays (4 in each coast) during winter (a total of 45 bays) were randomly selected. Different methodologies have been applied to assess viral contamination in oysters. Here we applied a method based on dissected-tissue analysis (3), which allowed us to have a more representative sample of the overall contamination, because oysters from the same bay and harvested at the same time were processed together and pooled.

Enteric caliciviruses (human and/or animal) were detected by RTPCR with broadly reactive and genogroup specific primers in oysters from 28 of 45 bays (62%) during summer or winter (Table 2.4 and 2.5). Simultaneous detection of human and animal enteric caliciviruses was observed in 5 samples (11%) (3 from OR, 1 from WA and 1 from DE); only human enteric caliciviruses were detected in 18 samples (40%) and only animal enteric caliciviruses were evident in 5 samples (11%). Other reports have shown a lower percentage of detection in areas authorized or non-authorized for harvesting. Human noroviruses were detected in 0-16% of samples collected from 20 different areas authorized for harvesting (European Community category A: less than 230 \( E. \ coli \) cells per 100 g of shellfish flesh in 90% of the samples; and B: less than 4,600 \( E. \ coli \) cells per 100 g of shellfish flesh in 90% of the samples) in the north and south of Europe (16). In France HuNoVs have been detected in 23% of samples collected from authorized harvesting areas (European Community category B) during a 3-year study, and in England, NoVs were detected in 27% of samples collected from a highly polluted area (34, 37). In a study performed in the US in 2003, 52% of samples previously depurated and relocated to a prohibited shellfish-growing area were positive for HuNoVs.
Our results show a higher percentage of positive samples than the values detected in other studies, although the samples came from approved shellfish-growing areas based on the criteria used for bacterial contamination (fecal coliforms level). This difference could be a consequence of the sampling, because in our case samples were collected only at two time periods and in other studies, samples have been collected during consecutive months including many negative samples that decrease the percentage of positives. Our results confirm that fecal coliforms are not an appropriate marker for viral contamination and that contamination by virus should be directly assessed until a good marker is found. Other studies have suggested that F-RNA bacteriophage could be used as a viral indicator. However this is still questionable because in some reports these phages correlate well with the presence of human enteric viruses, whereas in other reports the correlation is poor (10, 44).

Hybridization assays with HuNoV GI or GII probes were used to confirm RTPCR products and for grouping the strains into GI or GII. The HuNoV strains detected belonged to GII. However, hybridization failed in 11 of 20 RTPCR samples positive for HuNoVs, presumably because of differences in the genome segment used for these probes, as previously reported (60). Positive samples for HuNoVs GII after hybridization included two from the Gulf Coast (10G and 12G), 2 from the East Coast (5E and 11E) and 5 from the West Coast (1W, 2W, 3W, 10W and 11W). Sequence analysis of the 116 bp segment obtained with primer set p110-NI of samples from WA (3W), OR (1W), LA (12G), NY (5E) and MS (10G) showed the highest identity with the Norovirus GII/4 strain, particularly with the NoV 95/96-US subset and other Lordsdale strains, previously detected in oysters and implicated in diarrhea outbreaks in the U.S and distributed
However because the sequence analyzed was short, further studies are required to assess strain identity.

Interestingly, three samples (7E, 7G and 8G) were only positive by RTPCR with broadly reactive primer set NVp110-p290, but negative with all NoV genogroup specific primers (GI and GII). These 3 samples were also negative with primers designed for animal enteric caliciviruses. These results could be explained by the fact that NVp110-p290 can also detect SaVs (27). Although SaVs are not frequently associated with foodborne outbreaks (19), the possibility of water contaminated with enteric caliciviruses other than NoVs GII can not be dismissed. Most of the surveillance systems applied until now have only tested for NoVs, given their high association with foodborne outbreaks, but other members of the *Caliciviridae* family, i.e. SaVs, could also be present.

To our knowledge animal enteric caliciviruses have not previously been reported in shellfish. In our survey, animal enteric caliciviruses were detected by RTPCR and confirmed by hybridization or sequence analysis in oysters obtained from 10 of 45 bays distributed on the three coasts. Seven of 45 samples (16%) were confirmed as PoNoV positive after hybridization. The positive samples included six from the West Coast (CA, WA and OR) and one from the East Coast (DE). Sequence analysis of the 210 bp segment of these samples obtained with primer set PNV7-Bio/8, showed the presence of NoVs GII. In our study PoSaV was also detected in OR and BoNoV was detected in samples from OR and WA. The BoNoVs, PoNoVs and PoSaVs have been detected and characterized directly from cattle and swine samples, respectively in the US as well as in Europe and Japan (55, 57, 62). Phylogenetic analysis indicates that the BoNoVs and PoNoVs differ from HuNoVs, but they cluster in the NoV genus with BoNoVs in GIII
and PoNoVs in GII, the latter, the most frequently detected genogroup in humans. There is little data regarding interspecies transmission of enteric caliciviruses. Animal caliciviruses in the *Vesivirus* genus have wide host ranges and interspecies transmission has been documented (54). Although animal enteric caliciviruses have not been isolated from humans, human infection with NoVs related to BoNoV GIII was suggested by the presence of antibodies against BoNoV GIII/2 in veterinarians in The Netherlands (63). On the other hand, Oliver et al (47), and Han et al (23) have reported that bovine strains are unlikely to be a risk for humans, because they form a third genogroup distinct from the HuNoVs. However in the case of PoNoVs, phylogenetic analysis indicates that PoNoVs strains belong to, two PoNoV distinct clusters, 11 and 19, but also to cluster 18 that includes human GII strains (62), the most widely detected genogroup in humans, suggesting that under appropriate conditions interspecies transmission could occur (24).

Human and animal enteric caliciviruses were simultaneously detected in samples collected during summer in OR, WA and DE. The fact that two or more human viruses can be detected in the same sample has been described previously. In France the simultaneous detection of at least 4 different human viruses (NoVs, rotavirus, enterovirus and astrovirus) has been described for 10% of samples collected during 3 years (34). Coexistence of several NoVs GI and II and different genotypes within each genogroup, has been reported in oysters associated with foodborne outbreaks in Japan between December, 1998 and January 2001 (28). We focused our study not only on enteric caliciviruses, but also on both human and animal strains. The confirmed presence of two genogroup-related strains (human and/or animal) in the same sample introduces the potential risk for recombination among similar genogroup members in the human host.
Detection of HuNoVs and HuSaVs whose genomes derive from naturally occurring recombinants between members of the same (26, 29, 30) or different (5, 24) genogroup (intragenerogroup and intergenogroup recombination, respectively), the existence of chimeric BoNoVs within the NoVs GIII (23, 46) and the recent detection of PoNoVs that belong to GII of the NoV genus, suggest that co-infection with genetically closely-related human and animal enteric caliciviruses could result in genomic recombination between them (as part of the natural evolution of NoVs) and emergence of new strains relevant to the control of NoV outbreaks in humans.

The seasonal and geographical distribution of the positive samples showed a dynamic pattern. Human caliciviruses were not detected in 3 states (CA, SC and NJ), whereas samples from the other 10 states were positive in summer (54%, 18/33) and/or winter (42%, 5/12). Two different studies in Europe have suggested a seasonal distribution in the number of NoV-positive samples detected, with a larger number of positive samples during winter. Formiga-Cruz et al (16) indicated an increased detection of NoVs in samples collected during January and February, whereas in France, Le Guyader et al (34) showed an increased detection of HuNoVs in samples collected from November to January. We found a higher prevalence during summer, but without a significant correlation with season, perhaps because a large geographic area was covered by our sampling. Similar to our results, studies by Myrmel et al (44) of shellfish from the Norwegian Coast did not find a correlation between the presence of HuNoVs in shellfish and season.

Within each state, when calicivirus was detected, it was present in both seasons (WA, NY and ME), only in summer (OR) or only in winter (VA). In addition, some bays
were positive and others were negative in the same state at different times. As reported in France, HuNoVs were not always detected at the same site in every season. This observation may be partially explained by the presence of fecal waste from human associated recreation in the water, illegal dumping or accidental contamination of water with human waste with or without treatment (floods, etc) or contamination during harvesting by infected but asymptomatic workers.

Contrary to observations for HuNoVs, animal enteric caliciviruses were present only in 4 states (WA, OR, CA, DE). Also the seasonal distribution was less variable. Whereas PoNoVs were detected only in summer, samples positive for BoNoVs and PoSaVs were detected in winter. It is not surprising that most of these samples were from WA and OR, because these states have the highest livestock production among those included in this study. What is noteworthy is the distribution of positive samples in these states. In WA, HuNoVs were detected in samples from bays 6W and 3W, where PoNoV was also detected. But in winter HuNoVs were detected only in 3W and BoNoVs were detected in 6W. Also notable was the detection of enteric caliciviruses in OR, where HuNoVs and PoNoVs were detected by RTPCR, confirmed by hybridization and sequencing in the 3 bays under study, but in winter no HuNoVs or PoNoVs were detected, but PoSaVs and BoNoVs were present.

To our knowledge no data has been previously published confirming viral contamination in commercial shellfish harvesting areas representative of a range of geographical locations throughout the US coasts and during different seasons. Our results support previous reports that confirm direct enteric calicivirus contamination of the harvested seafood (38). Also the presence of enteric caliciviruses of animal origin in
shellfish with potential risk for humans has not been previously reported. To our knowledge this study is the first to demonstrate that both human and animal enteric caliciviruses are present in oysters from approved harvesting areas. Because these areas have been approved based on fecal coliform levels, a different marker needs to be applied to determine contamination by enteric viruses. The fact that these oysters were purchased from farmers, wholesalers and retailers, suggests a potential disease risk that requires confirmation by outbreak investigation. The simultaneous detection of human and animal enteric caliciviruses raises concerns for possible human infections or co-infection of humans with human and animal enteric caliciviruses, resulting in recombination and emergence of new strains relevant for the control of the disease.

2.6 ACKNOWLEDGMENTS

We are grateful to Drs Han and Wang for their helpful advice and comments. We thank Dr Estes and Dr Atmar (Baylor College of Medicine, Houston, TX) for the IC. We thank Ms Ana Azevedo for technical assistance. Sequencing was performed at the Plant-Microbe Genomics Facility of the Ohio State University.

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2.7 REFERENCES


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<th>State</th>
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<th>State</th>
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<td>Winter</td>
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<td>Winter</td>
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**TABLE 2.1:** States and number of bays sampled on each state between summer, 2002 and winter 2002-2003
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<th>Sense</th>
<th>Genus</th>
<th>Sequence (5'-3')</th>
<th>Location</th>
<th>Ref</th>
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</tr>
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</tr>
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</tr>
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<td>NoV</td>
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<td></td>
<td></td>
</tr>
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<td>Porcine</td>
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<td>SaV</td>
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<td>(62)</td>
</tr>
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<td></td>
<td></td>
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<tr>
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<td>PoSaV</td>
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<td>(61)</td>
</tr>
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* Nucleotide positions are in reference to Hu/NLV/Norwalk/68/US (M87661), except where indicated.
* Nucleotide positions are in reference to Hu/NLV/Lordsdale/93/UK (X86557).
* Nucleotide positions are in reference to Bo/NLV/CV95-OH/02/US (AF542083).
* Oligonucleotides are 5' biotin-labeled for detection by microwell probe capture hybridization assay.
* Y is C + T; R is A + G; M is A + C; D is A+T+G; W is A+T

**TABLE 2.2:** Primers and probes used to detect human and animal noroviruses (NoVs) and sapoviruses (SaVs) in oysters.
200 ul of each inoculum were mixed with 1.5 g of hepatopancreas
RNA from inocula were previously extracted and the highest positive dilution was determined by RT-PCR with specific primers for each strain.

<table>
<thead>
<tr>
<th>Inocula a</th>
<th>Strain</th>
<th>Dilution</th>
<th>RT-PCR b</th>
<th>Presence of PCR Inhibitors</th>
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<tr>
<td>Batch AH (high concentration)</td>
<td>HuNoV GII</td>
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<td>1:10000</td>
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<td>HuNoV GII</td>
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<tr>
<td>Batch CH (high concentration)</td>
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<td>1:10000</td>
<td>Protocol 1: +, Protocol 2: +, Protocol 3: +</td>
</tr>
<tr>
<td>Batch CL (low concentration)</td>
<td>BoNoV GIII</td>
<td>1:1000</td>
<td>1:100</td>
<td>Protocol 1: +, Protocol 2: +, Protocol 3: +</td>
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**TABLE 2.3:** Virus elution, concentration and RNA extraction protocols
Table 2.4: Number of bays positive by RT-PCR, Nested-PCR and hybridization for human caliciviruses during summer 2002 and winter, 2002-2003

<table>
<thead>
<tr>
<th>Coast</th>
<th>Human Calicivirus</th>
<th>Human Norovirus</th>
<th>RTPCR</th>
<th>Hybridization</th>
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<tr>
<td></td>
<td>GI</td>
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<td>Total</td>
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<tr>
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<td>2</td>
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<td>9</td>
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<tr>
<td>Gulf (n:13)</td>
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<td>2</td>
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<tr>
<td>Total (n:45)</td>
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<td>0</td>
<td>20</td>
<td>9</td>
<td>3</td>
<td>23</td>
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</table>

*a* Samples were positive with broadly reactive primers and/or genogroup II specific primers 

*b* Samples were positive by hybridization with probe GGII 

*c* Samples were only positive with broadly reactive primer sets p110/p290 or JV12Y/13I 

*n*: number of bays tested on each coast
<table>
<thead>
<tr>
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<th>Porcine Norovirus</th>
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<td>Hybridization&lt;sup&gt;c&lt;/sup&gt;</td>
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<sup>a</sup> Samples were positive with CBECU-F/R primers set  
<sup>b</sup> Samples were positive with PEC66-B/65 primer set  
<sup>c</sup> Samples were positive by hybridization with probes PoSapo 1A, 1B and 1C  
<sup>d</sup> Samples were positive with PNV7-B/8 primer set  
<sup>e</sup> Samples were positive by hybridization with probes PoNoro 1A, 1B and 1C

Table 2.5: Number of bays positive by RT-PCR and microplate hybridization for animal enteric caliciviruses sampled during summer, 2002 and winter, 2002-2003.
### TABLE 2.6: Geographical and seasonal distribution of oysters positive for human and/or animal enteric calicivirus from East, West and Gulf coast during summer, 2002 and winter 2002-2003.

<table>
<thead>
<tr>
<th>West coast (state)</th>
<th>Summer</th>
<th>Winter</th>
<th>East coast (state)</th>
<th>Summer</th>
<th>Winter</th>
<th>Gulf coast (state)</th>
<th>Summer</th>
<th>Winter</th>
</tr>
</thead>
<tbody>
<tr>
<td>1W (OR)</td>
<td>HuNoV&lt;sup&gt;c,d&lt;/sup&gt;/PoNoV&lt;sup&gt;d,e&lt;/sup&gt;</td>
<td>/-PoSaV&lt;sup&gt;h&lt;/sup&gt;</td>
<td>1E (ME)</td>
<td>HuNoV/-</td>
<td>1G (FL)</td>
<td>N/A</td>
<td>HuNoV/-</td>
<td></td>
</tr>
<tr>
<td>2W (OR)</td>
<td>HuNoV&lt;sup&gt;c&lt;/sup&gt;/PoNoV&lt;sup&gt;d,e&lt;/sup&gt;</td>
<td>/-BoNoV&lt;sup&gt;g&lt;/sup&gt;</td>
<td>2E (ME)</td>
<td>HuNoV/-</td>
<td>2G (FL)</td>
<td>N/A</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>3W (WA)</td>
<td>HuNoV&lt;sup&gt;c,d&lt;/sup&gt;/PoNoV&lt;sup&gt;d,e&lt;/sup&gt;</td>
<td>HuNoV/-</td>
<td>3E (VA)</td>
<td>/-</td>
<td>3G (FL)</td>
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<td>N/A</td>
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</tr>
<tr>
<td>4W (WA)</td>
<td>HuNoV/-</td>
<td>/-BoNoV&lt;sup&gt;g&lt;/sup&gt;</td>
<td>4E (NY)</td>
<td>HuNoV/-</td>
<td>4G (FL)</td>
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<tr>
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<td>5E (NY)</td>
<td>HuNoV&lt;sup&gt;c,d&lt;/sup&gt;/-</td>
<td>5G (LA)</td>
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<td>/-</td>
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<td>HuNoV/-</td>
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<td>7E (DE)</td>
<td>HuCV&lt;sup&gt;f&lt;/sup&gt;/-</td>
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<td>HuCV&lt;sup&gt;f&lt;/sup&gt;/-</td>
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<td>HuNoV/-</td>
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<td>8E (DE)</td>
<td>HuNoV&lt;sup&gt;c,d&lt;/sup&gt;/PoNoV&lt;sup&gt;d,e&lt;/sup&gt;</td>
<td>8G (FL)</td>
<td>HuCV&lt;sup&gt;f&lt;/sup&gt;/-</td>
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<tr>
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<td>/-</td>
<td>N/A</td>
<td>9E (NY)</td>
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<td>10W (OR)</td>
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<td>10E (SC)</td>
<td>/-</td>
<td>10G (MS)</td>
<td>HuNoV&lt;sup&gt;c,d&lt;/sup&gt;/-</td>
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<td>HuNoV&lt;sup&gt;c&lt;/sup&gt;/-</td>
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<td>HuNoV&lt;sup&gt;c&lt;/sup&gt;/-</td>
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<td>/-</td>
<td>12G (LA)</td>
<td>HuNoV&lt;sup&gt;c,d&lt;/sup&gt;/-</td>
<td>N/A</td>
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</tbody>
</table>

<sup>a</sup> Human calicivirus  
<sup>b</sup> Animal enteric calicivirus  
<sup>c</sup> Samples were positive by hybridization with probe PoNoro1A/B/C  
<sup>d</sup> Samples were sequenced and confirmed as Norovirus GIII  
<sup>e</sup> Samples were positive by hybridization with probe PoSapo1A/B/C  
<sup>f</sup> Samples were positive only by RTPCR with primers p110-p290  
<sup>g</sup> Samples were sequenced and confirmed as Norovirus GII  
<sup>h</sup> Samples were positive by hybridization with probe GGII
Dissected digestive diverticula

Artificial contamination
Fecal sample
(virus in high or low concentration)

Viral elution
PBS
Chloroform-butanol

Viral concentration
PEG-precipitation

Nucleic acid extraction
Protocol 1
Proteinase K
Phenol-chloroform
CTAB
Ethanol precipitation
RNA

Protocol 2
RNeasy Mini Kit
(Qiagen)
RNA

Protocol 3
Proteinase K
RNeasy Mini Kit
RNA

RT-PCR with specific primers for each enteric calicivirus strain

**Figure 2.1:** Viral elution, concentration and nucleic acid extraction strategies applied. a According to Atmar et al 1995 (3) with minor modifications, b According to manufacturer’s instructions.
Figure 2.2: Geographical and seasonal distribution of samples positive for human and/or animal enteric calicivirus during summer, 2002 and winter 2002-2003.  

**a. Summer 2002; b. Winter 2002-2003.**

- 🐰 HuNoV  🐏 BoNoV  🐃 PoNoV  🙃 PoSaV
- □ Negative  □ Not sampled
CHAPTER 3

EFFECT OF DIFFERENT SWINE WASTE TREATMENT TECHNOLOGIES ON DETECTION AND VIABILITY OF SWINE ENTERIC VIRUSES

3.1 SUMMARY

If not properly processed, enteric pathogens in animal waste can contaminate the environment and food. The persistence of pathogens in animal waste depends upon the waste treatment technology but little is known about persistence of swine viruses. Our objectives were to characterize the swine enteric viruses [noroviruses (PoNoV), sapoviruses (PoSaV), rotaviruses (RV)-group A, B and C] in fresh feces or manure and evaluate the effects of different candidate environmentally superior technologies (ESTs) for animal waste treatment on their detection. Untreated manure and samples collected at different stages during and after treatment were obtained from swine farms with conventional waste management (CWM) and 5 different candidate ESTs. The RNA from porcine enteric viruses was detected by RTPCR and/or seminested-PCR; PoSaV and RV-A were also assayed by ELISA. Cell-culture immunofluorescence (CCIF) and experimental inoculation of Gnotobiotic (Gn) pigs were used to determine RV-A/C
infectivity in post-treatment samples. The PoSaV and RV-A were detected in pre-treatment samples from each farm, whereas PoNoV and RV-C were detected in pre-treatment feces from 3/5 or 4/5 candidate ESTs, respectively. After treatment PoSaV RNA was only detected in the CWM, and not from the candidate ESTs. Rotavirus-A and C RNA was detected in 4/5 and 3/4 candidate ESTs after treatment, but infectious particles were not detected by CCIF, nor were clinical signs or seroconversion detected in inoculated Gn pigs. These results indicate that only RV-A/C RNA, but no viral infectivity was detected after treatment. Our findings address a public health concern regarding environmental quality surrounding swine production units.

3.2 INTRODUCTION

Increased animal production and decline in the numbers of producers in the U.S. have led to megascale livestock operations (39). The confinement of animals on relatively small land areas has resulted in the generation, accumulation and need for disposal of large amounts of animal wastes worldwide (6, 35). The causative agents of many infectious diseases are excreted by the fecal route, from animals with acute and chronic infections, but also sometimes from clinically healthy animals. In all types of livestock housing, waste material on site produces odors and contains antimicrobials, nutrients, organic matter and pathogens. Storage and treatment of this waste before land application are typically done in wastewater lagoons. However lagoon management presents significant concerns (18). With a greater opportunity for horizontal spread of infectious agents among closely confined animals, manure contains pathogens that can be
transmitted to other animals or to farm workers or to the public via contaminated meat products or water sources (12).

Environmentally superior technologies (ESTs) for animal waste treatment have been defined as those that are not only technically, economically and operationally feasible, but also are able to eliminate the direct discharge of animal waste to surface and ground water, and to reduce the levels of odors, ammonium, pathogens, nutrients and heavy metals. At least 18 candidate ESTs have been developed for treatment of animal wastes to reduce their impact on the environment, the food supply and public health (20). Several studies have been performed to determine the presence, persistence and rate of bacterial pathogen reduction in the newly developed treatments (6, 17, 24, 25, 33, 42, 47). However little is known about the presence and persistence of viruses in swine waste. In 2001 USEPA and USDA identified several infectious disease agents associated with manure, and the research needs to better document their presence and fate through different treatments (34). Among the most common causes of viral infectious diarrhea in pigs, porcine enteric caliciviruses [noroviruses (PoNoV), and sapoviruses (PoSaV)] and rotaviruses [(RV)-group A, B and C] are of particular concern because of their environmental stability, resistance to disinfectants and prolonged infectivity in feces.

The main contributors to foodborne illness are noroviruses (a genus in the Caliciviridae family) which are estimated to cause 82% of the illnesses caused by known pathogens (37). Porcine Norovirus was detected in Japan (36) and Europe (41) and recently in the U.S. (44). In Japan, PoNoV was detected in cecal contents of healthy pigs and in The Netherlands in fecal samples from 3- to 9-month-old healthy pigs. In the U.S., PoNoV was detected in fecal samples, but only from finisher pigs. Most positive samples
were from healthy animals suggesting that, as previously observed for human norovirus infections, asymptomatic shedding of PoNoV occurs in adults, thereby contributing to virus persistence in the field (46).

*Sapoviruses* (another genus in the *Caliciviridae* family) are also associated with gastroenteritis and play an important role in outbreaks of infantile and elderly gastroenteritis (16). The PoSaV has emerged as an important pathogen associated with diarrhea and subclinical infections among pigs. The PoSaVs were first discovered in U.S. in 1980 (31). Since then, PoSaVs have been detected in fecal samples from diarrheic or normal, nursing and post-weaning pigs and also from normal finisher pigs (46). However pigs in all age groups are seropositive for PoSaV antibodies, indicative of high exposure rates. These findings suggest that PoSaV may be a major cause of diarrhea, but subclinical PoSaV infections may also occur. Moreover, identification of genetically closely related caliciviruses in humans and animals, and studies of the seroprevalence and antigenic cross-reactivity between them, suggest possible interspecies or zoonotic transmission (10, 45).

Rotaviruses are the leading cause of acute viral gastroenteritis in the young of both avian and mammalian species, including pigs and humans. Group A, B and C rotaviruses infect pigs (32, 52), and both RV-A and non-group A are detected in the same herd. The RV-A infection accounts for 53% of preweaning and 44% of postweaning diarrhea in swine, whereas 89% of all rotavirus diarrhea in commercial pig operations have been attributed to RV-A (14, 48). The RV-B and RV-C have been identified in humans and pigs but in lower percentage (4.6-18.2% and 10.8-22.7%, respectively) (13, 21). In the U.S. RV-C was identified in fecal samples collected from finishing pigs with
diarrhea by IEM, cell-culture immunofluorescence and RTPCR (23). Furthermore, the finding of a low prevalence of antibodies to RV-C in humans and a higher prevalence of antibodies in pigs has led to the suggestion that RV-C could be a zoonotic infection (14). Likewise for RV-A, pig-like serotypes were detected from children and human serotypes have been shown to infect pigs (11, 32) confirming their interspecies transmission. Rotavirus is highly stable and it can survive in the feces as long as 7-9 months at room temperature (18-20°C)(51). Farm expansion, all-in all-out production, a non-previously circulating RV strain, and high population density are considered as the major risk factors that contributed to an outbreak (2, 7).

The objectives of this study were to characterize the occurrence of PoNoV, PoSaV, RV-A,-B and -C in fresh excreta and swine manure under defined animal waste treatment systems and to evaluate the effect of 5 different candidate ESTs for swine waste management on the detection of these viral pathogens.

### 3.3 MATERIALS AND METHODS

**Environmentally superior technologies:** Five different candidate ESTs and a conventional (lagoon) operation were evaluated: 1) Conventional waste management (CWM); 2) Aerobic Up Flow Biofiltration system (AUFBS); 3) Solid Separation/Constructed Wetland system (CWS); 4) Super Soil system (SSS); 5) In ground Ambient Temperature Anaerobic Digester and greenhouse system (ATAnD) and 6) High Rise Hog Building (HRHB). The CWM and technologies 2 to 5 were tested in North Carolina as part of the Agreement between the Attorney General of North Carolina and Smithfield Foods, Premium Standard Farms and Frontline Farmers to identify
alternative technologies to replace anaerobic lagoons (49, 50). The HRHB was developed as an alternative method for treatment of animal waste to produce composted solids without liquids (22). The system was originally built in Ohio and has been evaluated by researchers at the Ohio Agricultural Research and Development Center (OARDC).

**Site characteristics:** Seven swine production sites were selected for this study. Each site consisted of a medium (750 - 2499 pigs weighing over 55 pounds) or large (2500 or more pigs weighing over 55 pounds) concentrated animal feeding operation (CAFO) according to the U.S. Environmental Protection Agency definition (9), and similar to each other in terms of production phase (with the exception of ATAnD technology). When it was possible, technologies were evaluated at two different time points during a year. Site characteristics are summarized in Table 3.1.

**Samples:** Fresh swine fecal specimens were aseptically collected from 10 different spots along a zig-zag path through an animal pen or waste pit. To evaluate the effects of these technologies, fresh excreta as well as samples of manure and litter, pre- and post-treatment samples and samples at different steps following treatment were collected from each swine waste management system. Samples were collected at different treatment points according to the technology (Table 3.2), frozen and shipped overnight to our lab.

**Sample preparation and nucleic acid extraction:** Samples were diluted 1:10 in PBS (pH 7.4), homogenized and centrifuged for 30 minutes at 1,500 x g at 4°C. The supernatants were aspirated, aliquoted and stored at -20°C until analysis. Samples were tested by ELISA, RTPCR and/or seminested-PCR.
Nucleic acids were extracted with TRIZOL (Invitrogen) according to the manufacturer’s directions and the RNA was resuspended in 50 ul of DEPC-treated water. The presence of natural PCR inhibitors was tested by RTPCR using a competitive internal control (IC) and primers PEC-65 and PEC-66, specific for this IC as described below (43).

**Detection of PCR and RTPCR inhibitors in fecal samples:** One ul of a competitive IC was added to each RNA sample and RTPCR with primers PEC65/66, specific for this IC, was performed as follows: 42°C for 60 min and 94°C for 3 min, followed by 35 cycles of 94°C for 30 sec, 50°C for 60 sec and 72°C for 60 sec and a final extension of 10 min at 72°C. Amplified products were analyzed by electrophoresis using 2% agarose gel staining with ethidium bromide and visualized under U.V. If inhibition was detected, the RNA template was diluted 1:10 to overcome it and tested again.

**RTPCR for animal enteric calicivirus and seminested-PCR for rotavirus detection:** For animal enteric caliciviruses, RTPCR was performed with primers PEC66 (5’-GACTACAGCAAGTGGAATTCC-3’) and PEC-65 (5’-ATACACACAATTCATCCCCGTA-3’) for PoSaVs as previously described (19) and for PoNoVs, RTPCR was performed with primers PNV7 (5’-AGGTGGTGGCCGAGGAYC-3’) and PNV8 (5’-TCGCCATAGAAGTARAAG-3’) as previously described by Wang et al 2006 (43).

For RV-A, RTPCR was performed with primers sBeg-9 (5’-GGCTTTAAAA GAGAGAATTCCGTCCTGG-3’) and End-9 (5’-GGTCACATCATACAATTCTA ATCTAAG-3’) as previously described (15). The RTPCR products were diluted with DEPC-treated water (1:10) and a seminested-PCR was performed with primers GA75M
(5’-TAGGTATTGAATATACCACAA- 3’) and End-9 for 30 cycles at 94°C for 1 min, 52°C for 2 min and 72°C for 1 min and a final incubation at 72°C for 10 min. For RV-B, RTPCR was performed with primers 9B3 (5’-CAGTAACTCTATCCTTTTACC-3’) and 9B4 (5’-CGTATCGCAAATACAATCCG-3’) as previously described (5). For RV-C, RTPCR was performed with primers C1 (5’-CTGATGCTACTACAGAATCA-3’) and C4 (5’-AGCCACATAGTT CACATTTCATCC-3’). The RTPCR products were diluted 1:10 in DEPC-treated water and seminested-PCR was performed with primers C1 and C3 (5’-GGGATCATCCACGTCATGCG-3’) as previously described (14). Amplified products were analyzed by electrophoresis using 2% agarose gels staining with ethidium bromide and visualized under U.V. light.

**Detection of RV-A and PoSaV by antigen-ELISA:** Separate antigen capture enzyme-linked immunosorbent assays (ELISAs) were performed to detect RV-A and PoSaV as previously described (1, 19). Briefly for RV-A, half of the 96-well plates were coated with a gnotobiotic (Gn) pig hyperimmune anti-bovine RV-A serum (positive coating) or Gn pig RV-A antibody negative serum (negative coating) and incubated overnight at 4°C. After that, plates were washed with PBS/0.05%Tween and blocked with PBS/0.05%Tween/2% non-fat dry milk for 2 h at 37°C. After washing, each sample was added in duplicate to antibody positive and negative coated wells. Positive and negative controls were added to each plate (1). Plates were incubated for 1 h at 37°C. Hyperimmune guinea pig anti-bovine RV-A serum was added as secondary antibody. After incubation, anti-guinea pig IgG (H+L) peroxidase labeled (HRP) antibody (KPL, Inc, MD), was added to each well and incubated for 1 h at 37°C. Plates were developed by using ABTS 2-Component Microwell Peroxidase Substrate Kit (KPL, Inc, MD).
Absorbance values were read at 450 nm in an ELISA reader (Spectra Max 340PC, Molecular Devices, CA).

A similar procedure was followed for PoSaV (19). The 96-well plates were coated with hyperimmune guinea pig anti-PoSaV/Cowden strain virus-like particles (VLP) serum (positive coating) or pre-immunization guinea pig antibody negative serum and incubated overnight at 4ºC. Plates were washed, blocked and samples and controls were added and incubated after each reagent as above. Hyperimmune pig antiserum to PoSaV/Cowden strain was added as secondary antibody followed by, mouse monoclonal antibody to pig IgG conjugated to biotin. Streptavidin-HRP (Roche) was added and plates were developed with TMB 2-Component Microwell Peroxidase Substrate Kit (KPL, Inc, MD) and their absorbance values were read at 650 nm in an ELISA reader.

Detection of RV-A and RV-C infectious viral particles by CCIF in pre and post-treatment samples: A representative group of pre-treatment and all post-treatment samples that were positive by seminested-PCR for RV-A and/or RV-C were analyzed by cell culture immunofluorescence assay (CCIF) to detect infectious viral particles, as previously described (1, 38). Supernatants were diluted 1:10 in serum free minimum essential medium (MEM-E) and serial diluted 10-fold thereafter. Confluent monolayers of Ma104 cells were inoculated with 100 ul of each dilution in duplicate. Fifty ul of trypsin (2ug/ml) were added to each well and plates were centrifuged at 1,200 x g for 1 h at room temperature. After that, plates were incubated in a 5% CO2 atmosphere at 37ºC for 18 h. After incubation, monolayers were fixed with 80% acetone for 10 min and incubated overnight at 4ºC with fluorescein isothiocyanate (FITC)-conjugated
hyperimmune Gn pig anti-RV-C or FITC-conjugated hyperimmune bovine-RV-A serum. Fluorescing cells were visualized using a fluorescent microscope.

**Sequence analysis:** RTPCR and seminested-PCR products were purified from agarose gels by Geneclean kit as described by the manufacturer (Qiagen, MD). The DNA was sequenced directly using BigDye Terminator Cycle chemistry and 3700 DNA Analyzer (Applied Biosystems, Foster, CA). Sequence data were aligned using Lasergene software package (DNASTAR Inc., Madison, WI, USA) and compared with published sequences using Basic Local Alignment Search Tool (BLAST) and Clustal methods.

**Challenge of Gnotobiotic pigs with post-treatment samples:** To assess infectivity of post-treatment samples positive by seminested-PCR but negative by CCIF, hysterectomy-derived, colostrum-deprived 5-day-old Gn pigs were orally inoculated. Pigs were surgically derived and maintained in sterile isolator units (27). Selected post-treatment samples had the highest seminested-PCR titer (based on end-point titration) for RV-A or RV-C but were negative by CCIF for RV-A or RV-C, respectively. Samples were diluted 1:10 in MEM-E, homogenized, centrifuged and the recovered supernatants were filtered (0.2µ). Briefly, two Gn pigs were first orally inoculated with 5 ml of sample H-125 [RV-C, seminested-PCR (+), CCIF (-)]. Clinical parameters including diarrhea and fecal scores (0, normal; 1, pasty; 2, semiliquid; 3, liquid) were recorded. Fecal shedding of viral RNA was assayed by seminested-PCR from post inoculation day (PID) 0 to 5. At 7 PID, and in absence of clinical signs, both pigs were orally inoculated with 5 ml of sample H-134 [RV-A, seminested-PCR (+), CCIF (-)]. Clinical signs were recorded, whereas fecal shedding (viral RNA or virus) was assayed by seminested-PCR (RV-A and –C) and ELISA (RV-A) from PID 7 to 12. Serum samples were collected at
0, 14, 21, 31 PID and stored at -20°C until tested for antibodies to RV-A and RV-C by ELISA. Large and small intestine contents (LICs and SICs, respectively) were collected after euthanasia at PID 31.

**Detection of antibodies against RV-A and RV-C by ELISA:** For RV-A 96 well plates were coated with guinea pig hyperimmune anti-bovine RV-A serum and incubated at 4°C overnight (28). The plates were blocked with PBS/0.05% Tween/2% non-fat dry milk for 2 h at 37°C. The following reagents and samples were added in order: semipurified human RV-A Wa strain or mock cell culture supernatant; fourfold serial dilutions of serum; anti-swine IgG or IgM conjugated to biotin (KPL, Inc, MD); and (iv) Streptavidin-HRP (Roche). Plates were developed by using ABTS 2-Component Microwell Peroxidase Substrate Kit (KPL, Inc, MD) and absorbance values were read at 450 nm in an ELISA reader.

Similarly for RV-C, plates were coated with semipurified swine RV-C L1049 strain or mock cell culture supernatants, incubated overnight at 4°C and blocked with PBS/0.05% Tween/2% non-fat dry milk for 2 h at 37°C. Fourfold serial dilutions of serum, anti-swine IgG or IgM-HRP antibody (KPL, Inc, MD) were sequentially added. Plates were developed by using ABTS 2-Component Microwell Peroxidase Substrate Kit (KPL, Inc, MD) and absorbance values were read at 450 nm in an ELISA reader.

### 3.4 Results

**Occurrence of animal enteric viruses in fresh excreta:** Animal enteric caliciviruses and rotaviruses were detected by ELISA and different RTPCR and seminested-PCR assays. Porcine sapoviruses were detected in 59/61 (97%) samples by
RTPCR, ELISA or both techniques. Eight samples from different sites were sequenced confirming the presence of PoSaV GIII. Porcine noroviruses were detected in 12/61 (20%) samples by RTPCR (Table 3.3). Sequence analysis of samples collected in different sites confirmed the presence of PoNoV GII.

Samples were also tested for the presence of RV-A by ELISA and seminested-PCR. Forty one of 61 samples (67%) were positive for RV-A by seminested-PCR, or both ELISA and seminested-PCR. None of the samples were positive for RV-B by RTPCR. Rotavirus group C was detected in 27/61 (44%) samples by seminested-PCR (Table 3.4).

**Effect of different candidate ESTs for swine waste management on the detection of animal enteric viruses by ELISA, RTPCR and/or seminested-PCR:** PoSaV, RV-A and RV-C were detected in pre- and post-treatment samples, but PoNoVs were only detected in pre-treatment samples (Table 3.3,3.4). A total of 75 samples were collected at different steps during treatment. None of these samples were positive for PoNoV. However, 27/75 (36%) were positive for PoSaV by RTPCR, ELISA or both techniques. Thirty two of 75 (43%) samples were also positive for RV-A by seminested-PCR, or both seminested-PCR and ELISA. Rotavirus group C was detected in 41/75 (55%) samples by seminested-PCR. Only two post-treatment sample of 28 (7%) were positive for PoSaV by RTPCR, but RV-A and RV-C were detected in 14/28 (50%) and 12/28 (43%) post-treatment samples, respectively by seminested-PCR (Table 3.3,3.4).

With respect to the applied technologies, PoSaV and RV-A were detected in feces (pre-treatment samples) from control sites (CWM) and every site where a candidate EST was tested. However, PoNoV and RV-C were detected in pre-treatment samples from
those sites where CWS, SSS and HRHB (for PoNoV) or AUFBS, SSS, ATAnD and HRHB (for RV-C) were applied (Fig.3.1). Among the samples collected during treatment, PoSaV was not detected in CWS, or SSS. In the ATAnD technology, PoSaV was no longer detected after house effluents were treated in the anaerobic digester. In the AUFBS system, PoSaVs were detected in solids separated from the house effluent, but not in those liquids that were further processed. In HRHB, PoSaV was detected in the initial, but not final compost samples. Porcine sapovirus was detected in lagoon samples (post-treatment) in CWM, and in lagoon 3 (bypass) from AUFBS, but not in post-treatment samples from the 5 technologies.

The results obtained for RV-A and RV-C differed from those for PoSaV and PoNoV. In ATAnD technology, RV-A and RV-C were detected in effluents from the anaerobic digester, but they were not detected after Biofilter #1. Rotavirus group A was detected in post-treatment samples from CWM (lagoon samples), and also in post-treatment samples from CWS (storage pond) by seminested-PCR. In those sites where both, RV-A and RV-C were detected results were generally similar. In AUFBS, both viruses were detected by seminested-PCR in samples collected from lagoon 1, 2 and 3 at the end of the treatment. In SSS, samples from the bagged final product as well as the liquid obtained after phosphorus removal were positive for both viruses. Both viruses were also detected in the final compost produced after HRHB treatment.

Detection of RV-A and RV-C infectious viral particles by CCIF in pre- and post-treatment samples: A representative group of 25/41 pre-treatment samples positive by seminested-PCR for RV-A were tested by CCIF. Infectious RV-A particles were detected in 21/25 (84%) tested samples (Table 3.4). Positive samples belonged to the 5
ESTs tested and control sites (Fig.3.1). For RV-C, a subset of 17/27 pre-treatment samples positive by seminested-PCR were tested. Fourteen of 17 samples (82%) were positive by CCIF and belonged to AUFBS, SSS, ATAnD and HRHB (Table 3.4, Fig 3.1).

All post-treatment samples positive for RV-A or RV-C by seminested-PCR were assayed by CCIF. For RV-A, 4/14 (29%) post-treatment samples positive by seminested-PCR were also positive by CCIF. For RV-C, 2/12 (17%) post-treatment samples positive by seminested-PCR were also positive by CCIF. All CCIF-positive samples for RV-A and RV-C belonged to CWM (control sites) and lagoon 3 (bypass) in AUFBS (Table 3.4, Fig.3.1).

**Challenge of Gnotobiotic pigs with post-treatment samples:** To assess infectivity of post-treatment samples positive by seminested-PCR but negative by CCIF, two Gn pigs were successively inoculated with H-125 [post-treatment sample, RV-C seminested-PCR (+), CCIF (-)] and sample H-134 [post-treatment sample, RV-A seminested-PCR (+), CCIF (-)]. The H125 sample did not cause diarrhea (score 0-1) and RV-C RNA was only detected by seminested-PCR at PID1. At PID 7, and in absence of diarrhea, Gn pigs were inoculated with H-134. The H134 induced no diarrhea (score 0-1) and RV-A RNA was detected only by seminested-PCR one day after inoculation. Seroconversion to RV-A and/or RV-C was not detected (data not shown).

### 3.5 DISCUSSION

Land application of agricultural waste occurs worldwide, and pathogens present in manure can affect soil and water quality as well as the health of animals and humans working in livestock production. Different environmental factors affect the fate and transport of pathogens from waste into soil and water (12). Several technologies have
been developed to reduce the impact of animal waste in the environment. In this study we assessed the presence of five different enteric viruses after treatment of swine waste by 5 different candidate ESTs and in a conventional waste management operation (control).

Porcine sapoviruses and RV-A were detected in fecal samples collected from pigs in each site under study. However PoNoV was only detected in those sites where CWS, SSS and HRHB were tested, allowing us to evaluate only these systems for PoNoV. Similarly, RV-C was detected only in those sites where AUFBS, ATAnD, SSS and HRHB were evaluated. No differences related to seasonality or production size were detected (Fig.3.1).

Animal enteric caliciviruses cause gastroenteritis in calves and pigs (19, 40) but have also been isolated from healthy pigs (43, 44). The PoSaV has emerged as an important pathogen associated with diarrhea and subclinical infections among pigs of all ages. Their prevalence in feces varies between 21-100%, in samples collected from pigs with diarrhea (90-100%) and clinically normal (21-83%) (43). In our study, the PoSaVs were detected in 97% of pre-treatment samples by RTPCR, ELISA or both techniques, regardless of the type of swine production where the technology was applied. Porcine NoV were only detected in 3- to 9- month-old asymptomatic pigs in Japan, Europe and U.S., but their prevalence (0.5-20%) was much lower than PoSaV (36, 41, 46). In our study PoNoVs were detected only in 20% of pre-treatment samples. Compared to PoSaV, PoNoV have always been detected in clinically healthy finisher pigs suggesting that, as previously observed for HuNoV infections, asymptomatic shedding of PoNoV occurs in adults contributing to virus persistence in the field (30). Similar results were observed in our study, where PoNoVs were detected only in fecal samples from sites with finishing
pigs (CWS, SSS, HRHB), but not in samples collected in farrowing/gestation houses where the ATAnD technology was applied. However PoNoVs were not detected in AUFBS and CWM sites that also contained finishing pigs, which is in agreement with previous field studies that indicate that the prevalence of PoNoVs in the field is not as widespread for PoSaV.

Rotaviruses are ubiquitous in the environment. Rotaviruses-A, -B and -C infect pigs and both group A and non-group A can be detected from the same farm. Disease is influenced by pig age, management conditions and practices (52). Although disease is more frequently detected in 1- to 5-week-old pigs, RV can also cause diarrhea in feeder pigs (23). In our study RV-A and RV-C were detected in 67% and 44% of pre-treatment (feces) samples, respectively (Table 3.4). The RV-B was not detected in our study. This could be because of the absence of the virus, a lower shedding (3), or a test with low sensitivity (compared with the seminested-PCRs applied for RV-A and RV-C). Whereas one or all of these factors could be involved, the fact that all RVs have similar stability suggests that the effect of these candidate ESTs on the detection of RV-B (if the virus were present) will be similar to RV-A and RV-C.

Results differed using molecular techniques (RTPCR or seminested-PCR) and classical techniques such as ELISA for detection, and could be explained by differences in the target and sensitivity of each test. Regarding the former, RTPCR (or seminested-PCR) detects RNA (free, partially or surrounded by a protein core), whereas an ELISA detects complete virus or viral proteins (assuming that antigenic epitopes are intact). In addition, the sensitivity of the RTPCR and ELISA methods for PoSaV were determined based on 10-fold serial dilution of a PoSaV/Cowden cell cultured strain, ranging from 10^1
to $10^7$ TCID$_{50}$/ml. The lowest amount of PoSaV virus particles detected by ELISA was $10^5$, whereas RTPCR detected RNA in the sample containing $10^4$ TCID$_{50}$/ml (data not shown). Similarly for RV-A, sensitivity of RTPCR, seminested-PCR and ELISA were determined based on 10-fold serial dilution of RV-A/Wa cell cultured strain, ranging from $10^1$ to $10^8$ FFU/ml by CCIF. The lowest detectable amount of viral RV-A by ELISA was $10^4$ FFU/ml, whereas the lowest positive signal by RTPCR was from the sample containing $10^3$ FFU/ml and the seminested-PCR increased this sensitivity by 100 times ($10^1$ FFU/ml). Thus an RTPCR/ seminested-PCR-positive and ELISA-negative sample could have been the consequence of a partially or totally destroyed particles or more likely low virus concentrations. On the other hand an RTPCR-negative ELISA-positive sample could be a consequence of mutations in the sequence targeted by RTPCR primers that do not result in a change at the epitope (protein level) detected by ELISA. At the present RTPCR is the most widely used assay to detect viral enteric pathogens in clinical and environmental samples (8, 26).

The five technologies evaluated for treatment of animal waste showed different results. Whereas each one of the candidate ESTs were able to decrease the virus concentrations to undetectable levels for PoSaV and/or PoNoV using a sensitive assay such as RTPCR, not all of them were effective for RV-A and RV-C. In fact post-treatment samples from AUFBS (lagoon 1 and 2), CWS, SSS and HRHB were negative for PoSaV and PoNoV, but positive for RV-A and RV-C (except for CWS where RV-C was not initially detected). The differences observed could be because the seminested-PCR (RV-A and RV-C) has been shown to be more sensitive than RTPCR (PoSaV and PoNoV) as indicated before, or they could be due to an actual decrease in virus
concentrations for enteric caliciviruses, but not for RV. This latter is corroborated by other studies that indicate RVs are one of the non-enveloped viruses that are most resistant to inactivation in different animal wastes (29). Based on these findings, the ATAnD system was the only technology able to completely decrease RV concentrations to undetectable levels as measured by highly sensitive molecular techniques. Post-treatment samples from CWM (control) and AUFBS lagoon 3 (that receives manure directly from the barn, without treatment) were positive by molecular techniques for those virus initially detected at each site.

However none of the techniques mentioned earlier differentiate between infectious or non-infectious virus particles, which is very important to assess the risk of disease transmission from environmental samples. The detection of RNA in a sample does not always indicate presence of infectious virus. Although it is expected that free RNA will be degraded in the absence of a protective viral core protein, no information is available about the time required for this to occur. To assess infectivity we tested a group of pre-treatment and all post-treatment RV-A and/or RV-C seminested-PCR positive samples by CCIF. Pre-treatment samples were positive by CCIF indicating that infectious RV-A or RV-C particles were present before treatment. All post-treatment samples collected from each candidate EST, but not from CWM or AUFBS lagoon 3 (bypass) were negative by the CCIF infectivity assay for RV-A and RV-C, indicating the absence of detectable infectious particles using this assay. However there are two potential deficiencies in this assay for RV: first, the sensitivity of the test, and second, the fact that wild type RVs initially may not replicate efficiently in the MA104 cells used for CCIF assay. Regarding the latter, because pre-treatment samples coming from each site and
post-treatment samples from control sites were positive by CCIF, these findings suggest that although replication may not be very efficient, it was adequate for CCIF detection, as long as the virus concentration was high enough based on the test detection limits.

To determine if infectious particles were present at concentrations not detected by CCIF, we inoculated Gn pigs that should be the most sensitive indication of virus infectivity. One- to five-day-old Gn pigs develop profuse watery, yellow- to-white flocculent diarrhea 1-2 days after inoculation with RV. The clinical signs last up to 7 days and resolve in 7 to 14 days. Seroconversion to IgM and IgG in serum is detected at PID 7 and 21, respectively (52). Pigs recovered from RV infection are protected from a second infection by homologous, but not heterologous RV. Moreover, dual infection of calves with RV-A and RV-C has shown an increase in RV-C shedding (4). Under a natural scenario, RV diarrhea may be less severe than the experimental disease developed by Gn pigs because of the moderating presence of actively (by a previous infection) or passively (via colostrum or milk) acquired RV-specific antibodies (52). We inoculated Gn pigs with RV-A and RV-C seminested-PCR (+) CCIF (-) samples and observed the pigs for clinical signs for 31 days post-exposure. Neither diarrhea nor seroconversion for IgM or IgG were detected. Virus shedding was detected only at PID 1 by seminested-PCR, but it was not detected by ELISA. These results indicate that Gn pigs were not infected by post-treatment samples and suggest that they would not secrete infectious virus in the field.

Virus inactivation depends on many factors such as environmental temperature, pH and type of animal waste. Differences were also found in the various steps at which viruses were no longer detectable using molecular techniques. In samples collected in different steps during the ATAnD system, PoSaV was not detected after anaerobic
treatment, but RV-A and RV-C were detected at this step and samples from the following step (Biofilter #1) were negative. In the AUFBS system, solids separated from house effluents were positive for both PoSaV and RVs, but the liquid portion remained positive only for RVs. These differences could be consequences of the physical properties of each virus, the sensitivity of the assay or the fact that the inactivation time needed increases for a liquid vs a solid waste (29).

In summary, enteric viruses present in animal wastes can survive for long periods of time if proper treatment is not applied and they may constitute a public health concern in regard to the environmental quality surrounding swine production operations. This study presents, to our knowledge, the first evaluation of the impact of different candidate ESTs on virus detection in swine manure, and suggests that although only ATAnD can reduce virus concentrations to undetectable levels as evaluated by molecular techniques, all the technologies tested were effective in reducing virus infectivity when evaluated by virus infectivity assay (CCIF) and Gn pig inoculation. However these findings should also be evaluated in consideration of the ability of each technology to reduce the impact of other factors (organic and inorganic residues, bacterial pathogens, ammonia volatilization, etc) on the environment.

3.6 ACKNOWLEDGMENTS

We thank Lynn Worley-Davis and Dr Huawei Sun for collecting the samples at NCSU and OSU, respectively, and Robert Deearth for organizing and storing them at OSU. We also thank the North Carolina State University Animal and Poultry Waste Management Center where AUFBS, CWS, ATAnD and SSS technologies were tested,
and OARDC where HRHB technology was tested. Sequencing was performed at the Plant-Microbe Genomics Facility of The Ohio State University.

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3.7 REFERENCES


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### Table 3.1: Site characteristics

| EST                                      | Nº of sites | Type of farm | Nº of houses | Total Nº of pigs | Test  
|------------------------------------------|-------------|--------------|--------------|------------------|-------
| Conventional Waste Management            | 2           | Finishing    | 4-8<sup>b</sup> | 5000/7000        | 1<sup>f</sup> |
| Aerobic Up-Flow Biofiltration System     | 1           | Finishing    | 5            | 4000             | 2<sup>g</sup> |
| Constructed Wetland system               | 1           | Finishing    | 4            | 3500             | 1<sup>h</sup> |
| Super Soil system                        | 1           | Finishing    | 6            | 4400             | 2<sup>i</sup> |
| In ground Ambient Temperature Anaerobic Digester | 1   | Farrow to weaning<sup>a</sup> | 6<sup>c</sup> | 7000<sup>d</sup>          | 2<sup>j</sup> |
| High Rise Hog Building                   | 1           | Finishing    | 1            | 1000             | 2<sup>k</sup> |

<sup>a</sup> Sows and newborn up to three weeks old  
<sup>b</sup> Four houses in one farm and 8 houses in the other  
<sup>c</sup> Six houses include two farrowing and four gestation houses  
<sup>d</sup> Include 4000 sows and 3000 weans  
<sup>e</sup> Technologies were tested twice/year, in same or different farms, except for CWS  
<sup>f</sup> February or May  
<sup>g</sup> April and June  
<sup>h</sup> November  
<sup>i</sup> April and May  
<sup>j</sup> August and November  
<sup>k</sup> Jun to Sept; Oct to Feb
Table 3.2: Samples and point of collection on each system

<table>
<thead>
<tr>
<th>Waste treatment technology</th>
<th>CWM</th>
<th>AUFBS</th>
<th>CWS</th>
<th>SSS</th>
<th>ATAnD</th>
<th>HRHB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feces a</td>
<td>Feces a</td>
<td>Feces a</td>
<td>Feces a</td>
<td>Feces a,c</td>
<td>Feces a</td>
<td></td>
</tr>
<tr>
<td>House effluent</td>
<td>House effluent</td>
<td>House effluent</td>
<td>House Effluent</td>
<td>House effluent</td>
<td>Bedding</td>
<td></td>
</tr>
<tr>
<td>Lagoon b</td>
<td>Separated solids</td>
<td>Inner effluent</td>
<td>Homo tank</td>
<td>Digester</td>
<td>Initial compost</td>
<td></td>
</tr>
<tr>
<td>Equalization tank</td>
<td>Solid for separation</td>
<td>Separated liquids</td>
<td>Storage pond</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biofilter effluent</td>
<td>Inner effluent</td>
<td>Pre-Phosphorus Removal liquid</td>
<td>Biofilter #2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biofilter backwash</td>
<td>Outer effluent</td>
<td>Post-Phosphorus Removal liquid b</td>
<td>Green house b</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lagoon 1, liquids b</td>
<td>Storage pond b</td>
<td>Bagged Final Product Solid b</td>
<td>Tomato media b</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lagoon 2, biosolids b</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lagoon 3, barn b,d</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Pre-treatment samples  b Post-treatment samples  c Include 4 gestation and 2 farrowing houses  
d Lagoon 3 is not connected to the treatment technology (by pass) and it receives manure from another 4 houses with 4000 pigs  
§ Candidate waste treatments are: CWM, Conventional Waste Management; AUFBS, Aerobic Up-Flo\biofiltration System; CWS, Constructed Wetland system; SSS, Super Soil system; ATAnD, In-ground Ambient Temperature Anaerobic Digester; HRHB, High Rise Hog Building
Table 3.3: Detection of PoNoV and PoSaV in pre-, post-, and during treatment samples in different candidate ESTs

<table>
<thead>
<tr>
<th>Collecting time</th>
<th>PoNoV</th>
<th>PoSaV</th>
<th>RTPCR</th>
<th>RTPCR (^a)</th>
<th>ELISA (^b)</th>
<th>RTPCR and ELISA (^c)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nº of positive (%)</td>
<td>Nº of positive (%)</td>
<td>Nº of positive (%)</td>
<td>Nº of positive (%)</td>
<td>Nº of positive (%)</td>
<td>Nº of positive (%)</td>
<td>Nº of positive (%)</td>
</tr>
<tr>
<td>Pre-treatment (N=61)</td>
<td>12 (20)</td>
<td>19 (31)</td>
<td>14 (23)</td>
<td>26 (43)</td>
<td>59 (97)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment (N=75)</td>
<td>0 (0)</td>
<td>5 (7)</td>
<td>18 (24)</td>
<td>4 (5)</td>
<td>27 (36)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Post- treatment (N=28)</td>
<td>0 (0)</td>
<td>2 (7)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>2 (7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total (N=164)</td>
<td>12 (7)</td>
<td>26 (16)</td>
<td>32 (19)</td>
<td>30 (18)</td>
<td>88 (54)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Samples were positive by RTPCR with primers PEC66/65
\(^b\) Samples were positive by ELISA for PoSaV
\(^c\) Samples were positive by both ELISA and RTPCR with primers PEC66/65
### Table 3.4: Detection of RV-A, RV-B and RV-C in pre-, post-, and during treatment samples in different candidate ESTs

<table>
<thead>
<tr>
<th>Collecting time</th>
<th>Seminested PCR (^a)</th>
<th>ELISA (^b)</th>
<th>Seminested and ELISA (^c)</th>
<th>Total</th>
<th>CCIF (^d)</th>
<th>RTPCR</th>
<th>Seminested PCR</th>
<th>CCIF (^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nº of positive (%)</td>
<td>Nº of positive (%)</td>
<td>Nº of positive (%)</td>
<td>Nº of positive / Nº tested (%) (^e)</td>
<td>Nº of positive (%)</td>
<td>Nº of positive (%)</td>
<td>Nº of positive / Nº tested (%) (^e)</td>
<td></td>
</tr>
<tr>
<td>Pre-treatment (N=61)</td>
<td>39 (64)</td>
<td>0 (0)</td>
<td>2 (3)</td>
<td>41 (67)</td>
<td>21/25 (84)</td>
<td>0 (0)</td>
<td>27 (44)</td>
<td>14/17 (82)</td>
</tr>
<tr>
<td>Treatment (N=75)</td>
<td>29 (39)</td>
<td>0 (0)</td>
<td>3 (4)</td>
<td>32 (43)</td>
<td>NA</td>
<td>0 (0)</td>
<td>41 (55)</td>
<td>NA</td>
</tr>
<tr>
<td>Post-treatment (N=28)</td>
<td>14 (50)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>14 (50)</td>
<td>4/14 (29)</td>
<td>0 (0)</td>
<td>12 (43)</td>
<td>2/12 (17)</td>
</tr>
<tr>
<td>Total (N=164)</td>
<td>82 (50)</td>
<td>0 (0)</td>
<td>5 (3)</td>
<td>87 (53)</td>
<td>25/39 (64)</td>
<td>0 (0)</td>
<td>80 (49)</td>
<td>16/29 (55)</td>
</tr>
</tbody>
</table>

\(^a\) Samples were positive by seminested-PCR with primers GA75M/End-9  
\(^b\) Samples were positive by ELISA for RV-A  
\(^c\) Samples were positive by both ELISA and seminested-PCR with primers GA75M/End-9  
\(^d\) A set of pre-treatment samples and all post-treatment samples with a seminested-PCR positive result were tested by CCIF as indicated  
\(^e\) Percentage refers to the total number of samples tested by CCIF
Figure 3.1: Detection of PoNoV, PoSaV, RV-A, RV-B and RV-C in pre- and post-treatment samples as well as different steps following treatment in different candidate ESTs and CWM. □ Negative; □ Positive by RTPCR, seminested-PCR and/or ELISA; ■ Positive by seminested-PCR and CCIF
CHAPTER 4

CELLULAR GENE EXPRESSION IN LLC-PK CELLS INFECTED WITH POSAV/COWDEN STRAIN: GLOBAL MONITORING WITH MICROARRAYS

4.1 SUMMARY

Despite numerous attempts, Noroviruses and Sapoviruses can not be propagated in routine monolayer cell cultures with the exception of PoSaV/Cowden strain and a murine NoV (MNV-1). To better understand the interactions between PoSaV/Cowden strain and the susceptible host cells (LLC-PK cells), we used oligonucleotide microarray carrying 13,000 porcine sequences. We harvested virus- and mock-infected cells at two time points (4h and 8h post infection, PI) after PoSaV/Cowden infection according to a one step growth experiment. Based on the criteria of a ≥ 1.5-fold change, at 4h PI, 29 genes were differentially up-regulated (none were downregulated) and, at 8h PI, 83 genes were up-regulated and 7 were down-regulated in virus infected cells compared to mock-infected cells. Genes clustered mainly into classes involved in cell cycle, cell proliferation and apoptosis, protein metabolism, immune and stress response and signal transduction. To our knowledge this is the first study of the global cellular gene expression altered by calicivirus infection of cell cultures, and shows an overview of the
general changes in gene expression, that could serve as a foundation for further analysis at the functional level.

4.2 INTRODUCTION

Caliciviruses are small, non-enveloped, single-stranded, positive-sense RNA viruses. The icosahedral viral capsid (27-40 nm in diameter) is composed of a single major (VP1) capsid protein containing genomic RNA of 7-8 kb, excluding the poly A tract (29). Viruses in the Caliciviridae family are classified into four genera based on sequence identities and genome organization: Vesivirus, Lagovirus, Norovirus and Sapovirus and a potential fifth genus (Becovirus) (29, 56). Noroviruses (NoVs) and Sapoviruses (SaVs) cause diarrhea in humans and animals and are referred to as human or animal enteric caliciviruses (HECV or AECV, respectively) (29). Low infectious doses, prolonged asymptomatic shedding (28, 53), environmental stability (2) and strain diversity increase the risk of enteric calicivirus (ECV) infections.

Human NoVs (HuNoVs) are recognized as the major cause of epidemic nonbacterial gastroenteritis worldwide being responsible for 73% to 95% of the outbreaks, and half of all gastroenteritis outbreaks (6, 23, 24, 43). They are also recognized as the major cause of sporadic cases of gastroenteritis (1), and the single virus genus responsible for 50-66% of foodborne illness in the U.S. (62). The main challenge in ECV studies is the fact that, despite numerous attempts, NoVs [except for a Murine Norovirus (MNV-1)] and SaVs, with the exception of a Porcine Sapovirus (PoSaV) strain, can not be propagated in routine monolayer cell cultures (22, 63). Recently, it was reported that MNV-1 replicates in macrophages and dendritic cells derived from mice lacking the
signal transducer and activator of transcription 1 (STAT 1\(^{+/-}\)) (63). A 3-dimensional organoid model of a highly differentiated human intestinal epithelial cell line has been developed to assess HuNoV infectivity, but their ability to support in vitro propagation of high titers of HuNoVs is unclear (9).

Sapoviruses are also associated with gastroenteritis and play an important role in outbreaks of infantile and elderly gastroenteritis (29). The PoSaV was discovered in the U.S. in 1980 (55) and it has emerged as an important pathogen associated with diarrhea and subclinical infections among pigs of all ages (36, 45, 52, 60). The PoSaV/Cowden strain was propagated in a continuous porcine kidney cell line (LLC-PK), but successful propagation was only achieved by adding an intestinal content filtrate (IC), derived from uninfected gnotobiotic pigs to the cell culture medium (50). Replication of PoSaV/Cowden may initially depend on a cyclic AMP (cAMP) signaling pathway induced by IC because inhibitors of cAMP inhibited the effect of IC in a dose-dependent manner for up to 72hs (10). Also increased levels of Protein Kinase A (PKA) that could down-regulate interferon (IFN)-mediated STAT1 activation were observed. Bile acids were identified as active factors in IC essential for growth of PoSaV/Cowden in cell culture (11). Given the essential role of STAT1 for antiviral innate immunity mediated by IFNs, the authors proposed that its down-regulation by bile acids might allow PoSaV/Cowden replication in vitro. However, the fact that IC inhibits the activation of STAT1 by IFNs, with higher efficiency than individual bile acids, but induces lower increases in cAMP levels suggests that other components of the IC might inhibit STAT1 activation by other mechanisms or other undefined mechanisms mediated by IC or bile acids contribute to PoSaV/Cowden replication (11).
To gain insight into the global changes in cellular expression occurring in LLC-PK cells at early times after exposure to PoSaV/Cowden strain, we applied oligonucleotide microarray technology. We monitored expression levels of 13,000 porcine genes at two different time points after PoSaV/Cowden strain infection. By comparing the mRNA levels of virus-infected vs mock-infected cells, we identified altered expression of 29 different genes at 4h post infection (PI) and 90 genes at 8hs PI. This study provides new information to understand virus-host interactions and could serve as basis for future investigation of the genes and pathways involved in ECV in vitro replication and in vivo disease.

4.3 MATERIALS AND METHODS

Cells, virus and intestinal content filtrate (IC): The porcine kidney LLC-PK cells were grown at 37°C in 5% CO₂ incubator in Eagle's Minimum Essential Medium (E-MEM) supplemented with 5% fetal bovine serum. Cell culture adapted PoSaV/Cowden strain was propagated in LLC-PK cells in the presence of 3% IC in the medium (25, 50). Intestinal content were aseptically collected from uninfected Gn pigs, diluted 1:10 in E-MEM, clarified by centrifugation, filtered through 0.45µm and stored in aliquots at -20°C as previously described (25).

Virus inoculation: Confluent 3-4-day-old monolayers of LLC-PK cells grown in 6 well plates were used for virus inoculation. Cells were cultured as described above and infected at a multiplicity of infection (MOI) of 10 median tissue culture infectious doses (TCID₅₀) per cell, or with E-MEM for mock-infected cells for 1h at 37 °C. After that, virus- and mock-infected cells were washed and incubated in the presence of 3% IC at
37°C for the remaining time. High M.O.I was used to insure simultaneous infection in all cells. Cells were harvested at different times and subjected to experimental analysis.

**Immunohistochemistry (IHC):** Virus- and mock-infected cells were fixed with 3.7% formaldehyde at different times after inoculation, and permeabilized with 1% Triton X-100 in PBS. After washing with PBS, guinea pig hyperimmune antiserum to PoSaV/Cowden strain virus-like-particles (VLPs) and HRP-conjugated goat anti-guinea pig IgG (H+L) (KPL) were added successively and incubated for 1 hr at 37 °C. Cells containing virus antigen were detected by staining with aminothylcarbazole (AEC) diluted in acetate buffer and counted with a light microscope.

**One step growth curve:** To determine the post-infection time to harvest cells, in order to assess gene expression at early times during replication, we first performed a one step growth experiment for PoSaV/Cowden strain in LLC-PK cells. Virus inoculation was performed using confluent 3-4 day-old monolayers of LLC-PK cells grown in 6 well plates as noted above. Cultures were harvested at 1, 2, 4, 8, 12, 24 and 48hs post inoculation (PI) (Fig.4.1). Supernatants were collected at each point to determine extracellular virus concentrations and cells adhered to the wells were scraped from the surface, frozen and thaw three times to release virus and to determine intracellular virus concentrations. Virus titration was conducted by inoculation of serial 10-fold dilutions of supernatant and/or cells scrapped from the wells and resuspended in media, onto LLC-PK cells grown in 96 well plates. Plates were incubated 72h and virus presence was detected by IHC as described above. Titers were determined by Reed and Munch and expressed as TCID$_{50}$/ml. In addition virus infected cells were fixed and evaluated by IHC at each time point.
**RNA extraction:** Three independent experiments were performed for array hybridization at each time point. For each experiment, total RNA from two virus- or mock-infected wells were pooled. Cells were washed twice with PBS and total RNA was extracted from cells at 4h and 8h post-inoculation using an RNAeasy mini kit according to the manufacturer's protocol (Qiagen Inc.). Genomic DNA contamination was eliminated by treatment with RNase-free DNase (Qiagen Inc.) during RNA extraction. The yield and purity of each RNA sample were determined using an Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Palo Alto, CA) at the Center for Animal Functional Genomics, at Michigan State University.

**Microarray hybridization:** Total RNA (12ug) from each sample of virus- or mock-infected cells was reverse transcribed and labeled with Alexa Fluor® 647 (R) and Alexa Fluor® 555 (G) (Invitrogen Inc.) respectively, using SuperScript Indirect cDNA Labeling system kit (Invitrogen Inc.) with oligo(dT). Labeled cDNAs were purified using an S.N.A.P.™ column purification kit according to the manufacturer's instructions (Invitrogen Inc.). Purified Alexa 555- and Alexa 647-labeled cDNA were hybridized to the microarray slides at 42°C for 6h, followed by 35°C for 6h and 30°C for 6h in SlideHyb™ buffer #3 (Ambion Inc.) according to the manufacturer's instructions. The Porcine Long Oligo array was obtained from the Animal Functional Genomics Core Group at Michigan State University. It contains 13,297, 70-mer probes, single-spotted on glass slides. A total of 10,230 of these probes are designed against known genes or TIGR consensus sequences. The remainder represent EST sequences or clusters with at least one 3’ EST.
The mapped gene index sequences used for probe design are aligned against the set of human and mouse RefSeqs, known pig genes, and human and mouse Ensembl transcript sequences. Positive and negative control sequences are spotted multiple times.

After hybridization, microarray slides were washed with 2x SSC, 0.1% SDS at 37°C, 0.2x SSC, 0.1% SDS at room temperature and 0.2x SSC at room temperature and dried by centrifugation. Each slide was scanned with the ArrayWorx™ scanner (Applied Precision LLC.) and data (spot intensities and background intensities) from the slides were extracted using GenePix Pro 6.1 software (Axon Instruments, Union City, CA). To discard label-biased results, dye swap experiments where mock samples were labeled with Alexa Fluor®647 and virus samples were labeled with Alexa Fluor®555 were done.

Data analysis: Raw data were normalized using BRB Array tools (http://linus.nci.nih.gov/BRB-ArrayTools.html) developed by the Biometric Research Branch of the US National Cancer Institute, as well as MIDAS software version 2.19, available at the TIGR website (http://www.tigr.org/software/tm4/) (54). To determine differentially expressed genes, different software programs were applied. For data normalized by BRB Array tool, one class analysis at each time point was conducted using Significant Analysis of Microarray (SAM 3.0) (59). A calculated false discovery rate of <20% was used to assign significance and a threshold of 2 for changes in mRNA levels from infected cells relative to non-infected cells was set. Data normalized by MIDAS software was further analyzed by MeV software also available at the TIGR website.

Gene ontology (GO) analysis: Biological pathways were identified by EASE software package downloaded from http://david.niaid.nih.gov/david/ease.htm (19, 32). Highlight categories in GO structure included apoptosis, cell adhesion, cell
communication, signal transduction, cell differentiation, transcription, synthesis, transport and biochemical pathway, signal transduction, intracellular signaling cascade, protein folding, proteolysis, protein ubiquitination, metabolism, immune and stress response.

4.4 RESULTS

One step growth curve: To determine the course of LLC-PK infection with PoSaV/Cowden strain, and to select the time points for the gene expression study, we monitored the expression of PoSaV/Cowden strain structural proteins and virus titer at different time points after infection in a one step growth experiment (Fig.1). To do this, LLC-PK cells were infected with PoSaV/Cowden strain at an M.O.I. of 10 TCID$_{50}$/cell. At several times post infection, the extracellular virus and cell-associated virus titers were determined by titration. Virus structural antigens expression was monitored by IHC in situ (Fig 4.3).

Approximately 1-3 x $10^4$ TCID$_{50}$/ml of virus were found in both the supernatant (representing extracellular virus) and cell-associated fractions of infected cultures 1h after infection when the inoculum (virus or mock) was replaced by E-MEM with 3% IC. During the following 3h, the virus titer decreased in both the supernatant and cells. Between 4h and 12h after infection, extracellular and cell-associated titers increased almost exponentially. Cytopathic effects were first detected at 8h and increased until 24h PI when all cells were rounded and detached from the well surface. After 12h PI, virus concentrations in the supernatants increased more slowly and reached a maximum titer of about $3 \times 10^6$ TCID$_{50}$/ml from 24h to 48h PI. The eclipse phase was approximately 3h and the replication cycle appeared to be 10-12h.
Cells at each time point were assayed by IHC to detect the appearance of viral antigen (Fig 4.3). The results showed that between 1h and 2 h PI, some virus particles were attached to the cells. At 4h PI, viral capsid antigens were not detected in infected cells. At 8 h PI, PoSaV capsid antigens were detected in cells and detection was more marked at 12h PI. At the next time point (24h), no cells were detected attached to the wells, indicating that most cells were already infected and died. This result coincides with the plateau reached by the virus concentration in the supernatant at 24 h PI (Fig 4.2).

**Microarray analysis:** RNA was harvested from LLC-PK cells infected with PoSaV/Cowden strains at one early time point (4h PI) in virus replication when virus had entered the cells and the viral capsid antigen was absent (as monitored by IHC) suggesting active virus replication, and at a late point (8h PI) in infection when the production of progeny virions was in progress (as detected by IHC), but cell death was not observed. The mRNAs isolated at 4h and 8h PI were from two independent studies, each performed in triplicate. The integrity of mRNA was confirmed by gel electrophoresis and the electropherograms obtained using an Agilent 2100 Bioanalyzer. After hybridization, signal intensities were measured by Gene Pix Pro 6.1 and analyzed using BRB array tools as well as Microarray Data Analysis System (MIDAS).

Porcine SaV-infected cells were compared to mock-infected cells as control to identify differentially expressed genes by Significant Analysis of Microarray (SAM). One class analysis was performed for each data set (time point) to create a list of significantly regulated genes. A delta parameter that yields a high ratio of true positive to false positive was chosen for each data set. As the log₂ of R/G normalized ratio was analyzed, a 1.5 fold change was chosen. The SAM one class response data plot for each
time point (Fig 4.4 a and b) shows the calculated number of significant and predicted false significant genes (referred to as False Discovery Rate). Overall gene up-regulation predominated over gene down-regulation at 4h and 8h PI and changes were not label-dependent because the same results were obtained from a dye swap-experiment (data not shown).

At 4h PI, transcription of 138 genes were significantly up-regulated (Fig 4a). Of these significantly regulated genes, 109 were not further analyzed because they were unclassified (34 genes) or did not reach the minimum threshold established (75 genes). The remaining 29 genes, their score (by SAM), fold change and major functions are listed in Table 4.1. Up-regulated genes represented multiple biological processes. The GO annotation mapping demonstrated that these genes are involved in cell cycle and proliferation, transcription, protein synthesis and degradation, signal transduction, immune and stress response (Fig 4.5).

At 8 h PI, 229 genes were significantly regulated. Of these 206 genes were up-regulated and 23 were down-regulated (Fig 4.4b). Again of 206 up-regulated genes, 96 were unclassified and 27 did not reach the minimum threshold, leaving 83 up-regulated genes for further analysis. From 23 down-regulated genes, only 7 were analyzed further, because 12 were unclassified and 4 did not reach the minimum threshold. The remaining 83 up-regulated and 7 down-regulated genes and their major functions are listed in Table 4.2. Both, up- and down-regulated genes are involved in several biological pathways as indicated by the GO annotation mapping (Fig 4.5).
4.5 DISCUSSION

This work represents the first analysis of global gene expression in LLC-PK cells infected with PoSaV/Cowden strain. The experiments were designed to assess patterns of gene expression in LLC-PK cells that are used to propagate PoSaV/Cowden strain, the only enteric calicivirus that grows in routine monolayer cell cultures (50). A total of 138 and 229 genes were differentially expressed at 4h and 8h PI, respectively. The accuracy of these results is supported by the fact that experiments were done in triplicate, the results were analyzed by different microarray specific software and dye-swap experiments showed no dye-biased results.

Previous studies by Flynn et al (1988) showed that after a low passage number of PoSaV/Cowden strain, infected LLC-PK cell cultures developed CPE by 2 to 3 days PI and complete monolayer detachment occurred at 3-4 days PI (25). Latter, similar experiments by Chang et al (2002) at low M.O.I. (0.05) showed that CPE started at 30h PI, and by 72h, the cell monolayer was completely destroyed. However when the M.O.I. was increased to 0.5, the time before development of CPE was reduced to 16-20h and all cells were affected by 48h. Similar results were observed with cells transfected with PoSaV/Cowden RNA at low and high M.O.I. (10). In the case of other members of the Caliciviridae family such as FCV, development of CPE was observed at earlier times using M.O.Is. of 1-100. When CRFK cells were infected with FCV, CPE was observed at 5h PI, while cells became rounded and detached at 8-14h (47, 57). In our study, development of CPE using an M.O.I. of 10 was more rapid than previously described for PoSaV, but slower than for FCV. This could be a consequence of the use of more highly
adapted (higher passage in cell culture) PoSaV/Cowden strain compared with that originally described by Flynn et al. 1988. Also the one step growth experiment was performed at high MOI (10 TCID\textsubscript{50}/cell) to ensure that most of the cells were simultaneously infected and because of that, only one round of replication was measured (15) minimizing the possibility of simultaneously detecting genes that are regulated at different times during replication, making it impossible to determine which ones are the consequence of virus infection and which ones are the host response. In that way virus replication and expression of genes in infected cells will occur simultaneously in all cells. A similar approach was used to determine gene expression patterns in Caco-2 cells following RV infection (16) and in another study, to synchronize hepatoma cells before inoculation with parvovirus H1, allowing the study of time points where neither changes in cell cycle distribution nor CPE were detected (39).

We choose to analyze gene expression at 4h and 8h PI. This approach allows distinct molecular changes to be detected in infected cells at a time (4 or 8h PI) when little or no cytopathic effects were observed. At these times, the low virus titer detected and the absence of viral capsid antigens (as suggested by PoSaV capsid antigen-negative IHC) in infected cells suggests that the virus was uncoated and it is replicating. At 8h few extracellular virions were present and there was an increase of cell-associated virus as detected by titration, indicating the beginning of virus assembly and release from the cell (Figs 4.2 and 4.3). Similar studies were done to determine cell transcriptional responses to RV infection at early (1h PI) and intermediate (16h PI) time points during the replication cycle before cells were lysed at 24-48h PI (16).
At an early time PI, (4h), 29 genes with known function were up-regulated and none was down-regulated, whereas at 8h, 83 genes were up-regulated and 7 genes were down-regulated. The products of these genes cluster mainly in the following functional groups: cell cycle and cell-cell signaling; immune and stress response; protein metabolism; signal transduction, intracellular signaling cascade; synthesis, transport and biochemical pathway; metabolism and transcription (Tables 4.1 and 4.2). However many of these genes are multifunctional and are assigned to more than one functional class. Although understanding the relationship between the expression level of these genes and PoSaV replication requires further analysis, we discuss those genes clustered in each category with the highest differences (fold or SAM score) from the control and potentially important for PoSaV replication.

Cell cycle: At 4 h PI, we observed a major up-regulation of Integrin β4 (ITGB4, 2.2 fold, score 4.1) which encodes an integrin that is non covalently associated to transmembrane glycoprotein receptors. Integrin α6β4 signaling proceeds through Src family kinase (SFK)-mediated phosphorylation of the cytoplasmic tail of β4, recruitment of Shc, and phosphoinositide-3 kinase (PI-3K) (48). Integrins mediate cell-matrix or cell-cell adhesion, and cell growth. In addition, other studies have shown that α6β4 integrin is implicated in transducing signals from the extracellular matrix that control other cellular functions besides cytoskeleton organization. A proposed α6β4-mediated cascade seems to be mediated by the activation of Ras-MAPK pathways (49). Activation of α6β4 leads to recruitment of Shc, Grb2 and Sos, activation of Ras and stimulation of the MAPK, JnK and Erk signaling cascades (44). In agreement with this, we detected an increase in the
expression of G3BP2 (Ras-GTPase activating protein) at 4h PI and MAPK4K1 at 8h PI. An increase in ITGB4 may be a physiological response to PoSaV infection.

**Apoptosis:** Apoptosis is a highly regulated process that results in internal proteolytic digestion, cytoskeletal disintegration, metabolic derangement and genomic fragmentation (5). The apoptotic machine is sensitive to several varied agents by both internal sensors (intrinsic pathway-mitochondrial-dependent) and external stimuli (extrinsic pathway-death receptor-mediated). Cells undergoing apoptosis show characteristic changes in morphology, outer cell membrane composition, nuclear chromatin compaction and fragmentation (5). These changes have been well characterized in CRFK cells infected with FCV (47, 57), cells from rabbits infected with RHDV (34), and similar cellular reorganization has been observed in RAW264.7 cells infected with MNV-1, although the presence of apoptotic proteins has not been studied in the case of MNV-1 (63).

Although FCV infection of CRFK cells progresses more rapidly than PoSaV/Cowden infection in LLC-PK cells, both viruses showed similar characteristics related to cell culture infectivity. When CRFK cells were infected with FCV, increased viral protein synthesis was observed at 4h PI (47, 57), whereas we observed this at 8-12h PI (as indicated by positive IHC) in the case of PoSaV/Cowden and LLC-PK cells. Cytopathic and rounded-cells were observed at 5-8h PI in CRFK cells and detachment and lysis was observed at 8-14h PI. Infected LLC-PK cells became rounded at 12h PI and complete detachment was observed at 24h PI. The similarity in morphological changes between both infections, suggests that as FCV, PoSaV/Cowden triggers apoptosis in LLC-PK cells.
The cascade of events responsible for these morphological changes is executed by the caspase family (CASP-X) of cysteine aspartate proteases. These proteins are present in the cells as inactive precursors, requiring biochemical activation and allowing a rapid response to apoptosis inducing signals (31). Because cells trigger apoptosis in response to a virus infection, viruses have developed several mechanisms to block apoptosis and to preserve the cell for replication (5). However, apoptosis is induced by other viruses in late stages of replication as a mechanism for dissemination without initiation of a host response (58).

Sosnovtsev et al (57) observed binding of ANNEXIN V (as indicative of phosphatidylserine translocation, characteristic of the early stage of apoptosis) to the surfaces of CRFK cells and condensed chromatin simultaneously with rounded infected-cells at 5h PI with FCV. At 8h PI, DNA fragmentation as well as CASP-8, CASP-9 and CASP-3 were detected. The authors also showed that apoptosis is triggered by the de novo synthesis of virus proteins, because UV-inactivated FCV that retained its ability to bind to CRFK cells did not trigger apoptosis, and induction of apoptosis was blocked by cycloheximide (an inhibitor of protein synthesis in eukaryotic cells). Because we analyzed gene expression at earlier time points than the study by Sosnovtsev et al (57), before CPE and rounded-cells were observed, we would expect to find up- and down-regulation of genes that mediate cell rearrangement, caspase activation and apoptosis regulation. Accordingly, we detected up-regulation of ANNEXIN A2 (ANXNA2, 1.8-fold, score 4) at 4h PI. Members of the Annexin family, including ANNEXIN V, are calcium-dependent phospholipid binding proteins implicated in membrane related events along endocytic and exocytic pathways. We also found a slight up-regulation of S100A6,
ADD3, and CYCLIN 4. Although they did not reach the 1.5 threshold value (and because of that were not included in the final listing), all of them are implicated in the dynamics of the cytoskeleton regulation. Particularly members of the S100 family inhibit tubulin polymerization and cause disassembly of microtubules (20). If the increase in these mRNAs reflects an increase of these proteins, it is possible that these proteins are involved, together with the already characterized members of the ANNEXIN family, in cytoskeletal disorganization observed in early apoptotic events.

When the intrinsic pathway is triggered, signals from up-regulated sensors, such as p53 are propagated to the mitochondria via Bcl-2 family members (BH3 only) such as Bid, Bax and Bad located at the outer mitochondrial membrane and promote release of Cytochrom C (CytC). This will recruit Apaf1 and pro-CASP-9, which is cleavaged to the active CASP-9. The activation cascade follows by activation of CASP-3, CASP-7 and other substrates in the cell (5). Anti-apoptotic mechanisms to control this pathway and promote cell survival include Bcl-2 itself and Bcl-X\textsubscript{L} that target at the mitochondrial level and inhibitor of apoptosis (IAP) family members that function at the CASP level. In agreement with an intrinsic pathway, at 8h PI, we detected up-regulation of CCLN2 (Cyclin L2, 3.4 fold) and ZNHIT1 (zinc finger, 2.3 fold). Both up-regulate p53, but particularly CCLN2 is an RNA polymerase II-associated cyclin, whose over expression up-regulates p53 and Bax, and down-regulates Bcl-2, leading to apoptosis (64). In support of this, Natoni et al (47), observed that release of CytC correlates with translocation of Bax from the cytosol of CRFK FCV-infected cells to the mitochondria.

At the mitochondrial level, apoptosis is characterized by deregulation of respiratory chain activity [inhibition of NADH-ubiquinone-reductase (complex I), and
inhibition of electron transfer at the ubiquinone-CytC reductase complex (complex III)] (8). At 8h PI, we detected, strong down-regulation of NADH-Dehydrogenase-ubiquinone-1 β complex (NDUFB8, -3.4-fold, score 5) and simultaneously up-regulation of Ubiquinol-CytC reductase complex (UCRC, 2.2 fold, score 9.4). Although the protein expression of UCRC needs to be directly measured, there is the possibility that the cell responds to the release of CytC by increasing the expression of UCRC. Our results agree with the loss of mitochondrial membrane potential detected in CRFK cells infected with FCV (47).

We also found some evidence of anti-apoptotic mechanisms. Late in infection at 8h PI, BIRC3 is up-regulated and SRP54 is down-regulated. The activity of CASP family members is inhibited by a conserved family of Inhibitors of Apoptosis (IAP) originally detected in baculovirus and characterized by a ZINC-binding motif called BIR (Baculoviral IAP repeat) (14). The third BIR domain, BIR3, detected in our study inhibits the activity of CASP-9, but not CASP-3 or CASP-7. Higher levels of CASP-3, but lower levels of CASP-9, were detected by Sosnovtsev et al in CRFK FCV-infected cells. The authors indicated that this could be a consequence of cleavage of CASP-9 by CASP-3, or adequate concentration of CASP-9 as required. Our results suggest that it could also be a consequence of a counter-mechanism to block virus-induced apoptosis. Moreover, BIRC3, as well as TBKBP1 (up-regulated 3-fold at 8h PI) and CC2DIA (up-regulated 1.5-fold at 8h PI) may up-regulate NFκB and Jnk pathways to control apoptosis and favor cell survival (35).

Collectively, the cytopathic changes as well as the expression levels of several cellular genes, suggest that PoSaV/Cowden strain triggers apoptosis by the intrinsic
pathway at later stages in replication, similar to FCV. It is important to demonstrate that up-regulation of these genes results in activation of CASP-9 and CASP-3. Also, to know how the PoSaV/Cowden strain triggers this pathway requires further investigation. Other RNA viruses such as PV (4) and Avian encephalomyelitis virus (40) have been shown to induce apoptosis through this intrinsic pathway.

**Signal transduction:** Changes in the expression of several interconnected genes involved in different signaling pathways were observed at 4h and 8h PI. The c-FOS gene encodes leucine zipper proteins that can dimerize with proteins of the JUN family, thereby forming the transcription factor complex AP-1. A dual mechanism of control by mitogen-activated protein kinases (MAPKs) operates over c-FOS. Activation of extracellular signal-regulated kinases (ERKs) leads to c-FOS expression and the post-translational modification by the direct phosphorylation of the c-FOS (61). As such, the FOS proteins are part of the MAPK signaling pathways, and because of that, are also involved in Toll-like receptor, B and T cell receptor signaling and have been implicated as regulators of cell proliferation, differentiation, transformation and apoptotic cell death. As noted before, up-regulation of integrin α6β4, could mediate activation of MAPK and Erk pathways, and through this increase c-FOS phosphorylation. Increase in c-FOS (as a virus strategy or host response) may lead to apoptotic cell death, which has been observed in Gn pigs inoculated with HuNoVs, (13).

At 4h PI, we observed up-regulation of noncatalytic region of tyrosine kinase adaptor protein 2 (NCK2, 2 fold, score 3.1). The NCK2 is an adapter protein which associates with tyrosine-phosphorylated growth factor receptors or their cellular substrates. The protein has no known catalytic function, but it has been shown to bind
and recruit various proteins involved in the regulation of receptor protein tyrosine kinases. Among the proteins that interact with NCK2 is PKN2 that belongs to the serine/threonine protein kinase family (PKC subfamily), also up-regulated at 4h PI (1.6 fold, score 6) (7). Recently, an alternative mechanism for inhibition of IFNα has been described (21). Du et al 2005 demonstrated that IFNα signaling is inhibited by a rapidly inducible pathway that requires PKCβ and SHP-2. Although we did not detect up-regulation of SHP-2 (perhaps occurring before 4h PI), we hypothesize that it was up-regulated, because up-regulation of MAPK4K1 by MAPK pathways requires SHP-2. Together, up-regulation of PKN2 (PKC subfamily) and SHP-2, could be a second strategy to down-regulate STAT signaling, and therefore IFNα.

Finally, also at 4h PI, a small GTPase activating protein SH3 domain-binding protein 2 (G3BP2) was up-regulated (1.6 fold, score 4.1). Functionally, this gene has been tested for association to cytoplasmic sequestering of NFκB, and Ras protein signal transduction (51). At 8 h PI, BCCIP was the highest up-regulated gene (3.6 fold). It is a cofactor for BRCA2 in tumor suppression, and modulator of CDK2 kinase activity via p21 (46).

**Immune response:** Curiously, we did not find up-regulation of the expression of genes related to the immune response, such as Interferon inducible genes (IIG), which are usually induced by other viruses (26). This may be because these genes were up-regulated at early times PI (1-2h) that were not assessed in our study, or because the virus down-regulates IFN responses early in infection. Chang et al (2004) demonstrated that successful propagation of PoSaV/Cowden strain in LLC-PK cells required down-regulation of IFN-mediated STAT1 activation. Similarly Wobus et al (2004) reported that
MNV-1 replicates in macrophages and dendritic cells derived from mice lacking STAT1 (63). The Proteasome activator unit 1 (PSME, implicated in immunoproteasome assembly and required for efficient antigen processing) and IRF5 were both up-regulated at 8h PI. The highest up-regulated gene at 8h PI, MAP4K1 (3.1 fold) plays a role in the response to environmental stress and appears to act in several pathways (MAPK Signaling Pathway, Signaling of Hepatocyte Growth Factor Receptor) and processes (activation of JNK activity, activation of MAPKKK activity, peptidyl-serine phosphorylation, protein kinase cascade, response to stress, protein amino acid phosphorylation) (33, 37). Although further investigation is required, the late up-regulation (8h PI) of MAP4K1, PSME, IRF5, the ability of G3BP2 (up-regulated at 4h) to bind and sequester NFκB, and the absence of other intermediates in IFN responses suggest a block or delay in the IFN response to virus infection. It has been shown that African swine fever virus, inhibits IFN production by blocking of the NFκB pathway (38). Interestingly, the activation of IRF-5 has been shown to be specific for different virus types, because it was detected in cells infected with NDV, but not Sendai virus (3).

**Protein metabolism:** Most of the genes related to protein metabolism and up-regulated play a role in catabolism. CATHEPSIN L is a lysosomal cysteine proteinase that plays a major role in intracellular protein catabolism. Several up-regulated genes at 4h PI (BARD1, CUL7, FZR1, Usp43, and UBE2D4) mediate the ubiquitination and subsequent proteosomal degradation of target proteins.

Five genes directly involved in protein synthesis were up-regulated at 8h PI. These include eukaryotic translation initiation factor 5B (MAP7D1, 4.1-fold, score 6.2), component of ribosome (RPL18, 1.8-fold, score 3.2), mitochondrial ribosome
components (MRPS9, 1.8 fold, score 4.6; MRPL24, 1.3-fold, score 4.2) and DEAD (Asp-Glu-Ala-Asp)/H Box polypeptide 49 (DDX49, 2.7-fold, score 3.9). No other elongation factors, tRNA synthases or intermediates required for translation were detected. Rotavirus infection of Caco-2 cells up-regulates the expression of six eIFs, four tRNA synthases and four DEAD box proteins (16), whereas PV induces up-regulation of PABP and cellular ribosome subunit S24 variant 2, among others in human fibroblasts (41, 42).

Two explanations are possible for the low number of genes up-regulated and related to translation. It is possible that other genes involved in translation regulation were up-regulated at earlier times which were not examined in this study. But it is also possible that, like other members of this virus family, PoSaV achieves viral protein translation by a different mechanism that does not require up-regulation of other factors. Initiation of translation of noroviruses, vesiviruses and lagoviruses depends on the VPg protein covalently linked to the 5’end of the viral genome, and latter linked to subgenomic RNA, for synthesis of structural proteins (30). In vitro experiments demonstrated that VPg binds eIF3, and directly or indirectly through its interaction with eIF3, it also binds eIF4G1 and the 40S ribosomal subunit (17). The same interactions were observed in vitro and in cells (RAW 264.7 cells) infected with MNV-1 (18), and they were also essential for FCV replication, both requiring the presence of eIF4A (helicase component) (12, 27).

In summary, this study has led to the identification of cellular genes whose expression is altered at early (4h) and late (8h) times during PoSaV infection of LLC-PK cells. Although the mechanisms are unknown, the data shows that most of the genes are up-regulated. A challenge for the future will be to determine which of these changes are
induced by the virus and which are part of the host response to the virus. The up-regulation of different proteins with opposite actions, such as Cyclin L2, G3BP2 and BIRC3, could be a consequence of the host response to the virus infection. Moreover further investigations are required to assess the relevance of these genes to the PoSaV life cycle. Our results have some limitations. Although the experiments have been replicated, dye-swap experiments have been included and different software analysis has been applied, microarray is a semiquantitative assay whose results must be confirmed by rt-RTPCR. It will also be of interest to determine gene expression at 1h or 2h PI, to determine if the virus shuts down or only delays the host antiviral response. Our data provide a basis for further studies of PoSaV at the functional level to understand the mechanisms of infection, with potential extension of the findings to the Noroviruses.

4.6 ACKNOWLEDGMENTS

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**Figure 4.1: One time growth experiment.** LLC-PK cells were infected with PoSaV at a MOI of 10 TCID$_{50}$/cell. Cells were incubated 1h at 37ºC. Medium was replaced with fresh medium containing 3% intestinal content. Supernatant and cells were harvested at different time points and virus yield was determined by IHC.
Figure 4.2: One time growth curve of PoSaV/Cowden strain: The extracellular (◊) and cell-associated virus (□) were titrated by immunohistochemistry (IHC).
Figure 4.3: PoSaV Capsid expression in LLC-PK cells: At different time points after infection, the supernatant was removed and cells were fixed. After incubation with guinea pig hyperimmune antiserum to PoSaV/Cowden strain-VLPs and HRP-conjugated goat anti-guinea pig IgG (H+L), virus structural antigen was detected by staining with AEC and observed using a light microscope. No cells were attached to the wells at 24h and 48h post infection. Representative pictures at each time point are shown.
Figure 4.4 SAM plot: One class analysis of LLC-PK cells at 4h (a) and 8h (b) after infection with PoSaV/Cowden strain. The three arrays at each time point were subjected to SAM one class analysis. Threshold and delta parameters were chosen to limit the field and calculate significantly regulated genes. Dots in red are genes up regulated and down regulated are in green.
Figure 4.5: Biological process gene ontology (GO) classification of differentially expressed mRNA. At 4h PI (□) 29 genes were only up regulated; at 8h PI, 83 genes up-regulated (■) differed from those down-regulated (7).
### Table 4.1: Genes differentially expressed (≥ 1.5 fold) in LLCPK cells infected with PoSaV/Cowden strain (4 h PI)

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Table 4.2: Genes differentially expressed (≥ 1.5 fold) in LLCPK cells infected with PoSaV/Cowden strain (8 h PI) (continued)
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**Metabolism**

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**Protein metabolism (protein folding, proteolysis, protein ubiquitination)**

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**Synthesis, transport and biochemical pathway**

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LIST OF REFERENCES


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