PATHOGENESIS OF HUMAN NOROVIRUS IN GNOTOBIOTIC PIGS

DISSERTATION

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By

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ABSTRACT

Noroviruses (NoVs) comprise a genus of the *Caliciviridae* family. The importance of NoVs as causative agents of gastroenteritis in humans has been underestimated for decades because of the lack of sensitive detection methods. It is still unclear whether this is a group of viruses that is emerging or if recent advances in molecular techniques are just unveiling their constant role as enteric pathogens. The NoVs are associated with high morbidity, low mortality rates and their clinical manifestations are usually mild and self-limiting in otherwise healthy individuals. Mainly immunocompromised individuals as well as the elderly or persons in the military under stressful conditions may have complications that require medical intervention. Nevertheless, losses of productivity, consumer decline in purchase of targeted foods after an outbreak and the costs of handling such outbreaks are considerable. There is a higher risk of NoV infection in communal settings such as day-care centers, schools, nursing homes, hospitals, cruise ships and also in the military. In addition, NoVs have been classified as biological pathogens class B by the Biodefense Program at the National Institute of Health. Sporadic cases of NoV are also common but the source of the virus
remains unclear. Although the NoV infectious dose is low and these viruses are resistant in the environment, the role of reservoirs such as asymptomatic individuals as well as domestic animals is under study. There is extensive genetic diversity in this virus family presenting difficulties not only for diagnoses and for understanding their epidemiology but also for designing prevention strategies. Moreover, most NoVs (except murine NoV) do not grow in cell culture; thus little is known of their replication cycle. These viruses cannot be amplified or titrated \textit{in vitro} and neutralizing antibodies cannot be defined. In the last 15 years greater numbers of NoV genomic sequences have been deposited in Genbank. Consequently, expression of recombinant capsid proteins that self-assemble into virus-like particles (VLPs), and construction of infectious replicons has permitted limited \textit{in vitro} studies of the antigenic relationships, their prevalence, the host’s immune response and the biology of these viruses. Recently, a murine NoV was identified that grows in cell culture, but the clinical disease is not gastroenteric but systemic in mice. Therefore, this is not a good surrogate virus to study the pathogenicity of enteric NoVs. Although the bovine NoVs that belong to a separate genogroup (GIII) can be studied in calves, an animal model to study the pathogenicity of human NoVs \textit{in vivo} is still lacking.

The NoVs contain single-stranded RNA of positive polarity in which mutations and recombination have been described. In general, RNA viruses evolve quickly due to genetic drift as a consequence of point mutations caused by a viral RNA polymerase that lacks proofreading activity and also, even more rapidly, by genetic shift due to recombination. Multiple NoV strains are present in water, food and the environment and mixed infections are common. This group of viruses has the potential of becoming a
more serious problem to public health. If we consider the fast evolution rate and observe what has happened to members of other genera in the calicivirus family: a vesivirus originally infecting marine mammals has shown a broad host range infecting humans as well as cattle, pigs, monkeys, fish and reptiles; some strains of feline calicivirus (another vesivirus) that normally cause cutaneous and respiratory disease in cats have mutated into highly pathogenic strains that cause systemic lethal disease in cats; and lagoviruses that infect rabbits and hares utilize histo-blood group antigens as receptors and cause a lethal hemorrhagic syndrome. Human NoVs also bind to histo-blood group antigens that are present in most human tissues; these antigens are also present in domestic animals and closely related NoV strains have been isolated from cattle and swine. Thus, surveillance and a better understanding of NoVs are necessary in order to evaluate the zoonotic potential of animal NoVs, be prepared for the eventual appearance of a new more virulent strain and to control the existing strains.

The first objective was to adapt a human NoV strain to replicate in gnotobiotic (Gn) pigs. An animal disease model to study the pathogenicity of human NoVs is critical for future evaluation of immune responses and vaccine efficacy. In addition, the role of pigs as a reservoir for human strains would be indirectly assessed. We identified a human GII cluster 4 NoV strain that replicated in Gn pigs causing mild diarrhea and brief fecal shedding in most pigs, but as described in humans, some pigs were resistant to infection.

Thus, the second objective was to determine if the pigs’ histo-blood group antigens (closely related to type A/H human antigens) expressed on buccal and intestinal tissues play a role in human NoV pathogenicity in swine as they do in humans. In an in
vitro study, certain human GI and GII NoV VLPs bound to these tissues of the A or H phenotype pigs. The A/H expression and the VLP binding patterns were compared. Human NoV VLPs of different clusters bound to tissues of A and H phenotype with different patterns. In an in vivo study, the A/H phenotype of 65 Gn pigs inoculated with human NoV GII/4 was determined and this data was compared to the infection outcome by NoV shedding or seroconversion for the acute and convalescent pigs, detected by RT-PCR and by antibody ELISA, respectively. The expression of A/H antigens in intestinal tissues of Gn pigs was associated with human GII NoV infection.

In summary, our studies were the first to compare the histo-blood group antigen expression in the pig to the NoV infection outcome and to show that the Gn pig is a good model to study the pathogenesis of human NoVs.
Dedicated to Darren, my family and my friends

Thanks for their love, patience, understanding and encouragement

through all these years
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LIST OF ABBREVIATIONS

Alanine aminotransferase (ALT)
3-Amino-9-ethylcarbazole (AEC)
Basic local alignment search tool (BLAST)
Binary ethylenimine (BEI)
Bovine enteric calicivirus (BEC)
Bovine serum albumin (BSA)
Canine calicivirus (CaCV)
Center for disease control and prevention (CDC)
Centimeter (cm)
Cesium chloride (CsCl)
Cetyltrimethylammonium bromide (CTAB)
Cholera toxin (CT)
Colostrum deprived (CD)
Copy DNA (cDNA)
Cyclic AMP (cAMP)
Cytotoxic T lymphocyte (CTL)
Diethyl pyrocarbonate (DEPC)
Electron microscopy (EM)
Encephalomyocarditis virus (EMCV)
Enzyme-linked immunoassay (ELISA)
European brown hare syndrome virus (EBHSV)
Feline calicivirus (FCV)
Genogroup (G)
Gnotobiotic (Gn)
Gram (g)
Guanidinium thiocyanate (GTC)
Hemagglutination (HA)
Hemagglutination inhibition (HI)
Heteroduplex motility assay (HMA)
High performance liquid chromatography (HPLC)
Histo-blood group antigens (HBGA)
Horseradish peroxidase (HRP)
Hour/hrs (h/hrs)
Human immunodeficiency virus (HIV)
Human Norovirus (HuNoV)
Immune electron microscopy (IEM)
Immunofluorescence (IF)
Immunoglobulin (Ig)
Immunostimulating complexes (ISCOM)
Inducible nitric oxide synthase (iNOS)
Institutional laboratory animal care and use committee (ILACUC)
Interferon (IFN)
Intestinal content (IC)
International Committee on the Taxonomy of Viruses (ICTV)
Internal ribosomal entry site (IRES)
Kilo base (kb)
Kilo Dalton (kDa)
Lewis antigens (Le)
Litre (l)
Logarithm (Log)
Messenger RNA (mRNA)
Microgram (μg)
Millijoule (mJ)
Milliliter (ml)
Minimal essential media (MEM)
Mink calicivirus (MCV)
Mink enteric calicivirus (MEC)
Monoclonal antibody (MAb)
Multiplicity of infection (MOI)
Murine Norovirus 1 (MNV-1)
Mutant *Escherichia coli* heat-labile toxin (mLT-R192G)
Nanogram (ng)
Nanometer (nm)
Nebraska/80/US bovine calicivirus (NB)
Newbury agent-1 (NA-1) and 2 (NA-2) bovine calicivirus
Nonsecretor (Se−)
Norovirus (NoV)
Nucleic acid sequence-based amplification (NASBA)
Nucleoside triphosphate (NTP)
Nucleotide triphosphatase (NTPase)
Open reading frames (ORF)
Parts per million (PPM)
PBS tween buffer (PTB)
Phosphorodiamidate morpholino oligomers (PMO)
Potassium phosphate sodium chloride buffer (PBS)
Potassium phosphate buffer (PPB)
Polyethylene glycol (PEG)
Polymerase chain reaction (PCR)
Porcine enteric calicivirus (PEC)
Post-inoculation day (PID)
Protein kinase A (PKA)
Protein kinase RNA-activated (PKR)
Protruding (P) domain of the capsid
Rabbit hemorrhagic disease virus (RHDV)
Radio immunoassay (RIA)
Recombinant Identification Program (RIP)
Red blood cells (RBC)
Reverse transcription (RT)
RNA-dependent RNA polymerase (RdRp)
Room temperature (Rt)
San Miguel sea lion virus (SMSV)
Sapovirus (SaV)
Seconds (Sec)
Secretor phenotype (Se+)
Shell domain (S domain) of capsid
Signal transducer and activator of transcription 1 (STAT-1)
Single stranded RNA ssRNA
Small, round-structured viruses (SRSV)
Sodium dodecyl sulfate (SDS)
Solid phase IEM (SPIEM)
Terminal deoxynucleotidyl transferase (TdT)
Tetramethylbenzidine (TMB)
T helper 1 lymphocyte (Th1)
Transmission electron microscopy (TEM)
Tris sodium chloride calcium chloride buffer (TNC)
Tumor necrosis factor (TNF)
TdT-mediated dUTP nick end labeling (TUNEL)
Untranslated region (UTR)
Ultraviolet (UV)
Uracyl –N-Glycolase (UNG)
Venezuelan equine encephalitis (VEE)
VEE replicon particles (VRPS)
Vesicular exanthema of swine-like virus (VESV)
Virus-like particles (VLPs)
Virus protein genome-linked (VPg)
Virus structural protein 1 (VP1)
Weak secretor phenotype (Sew)
CHAPTER 1: LITERATURE REVIEW

HUMAN NOROVIRUS AND OTHER MEMBERS OF THE CALICIVIRIDAE

1.1 History

Nonbacterial gastroenteritis outbreaks have been described since the beginning of the 20th century. Reports of nonbacterial vomiting and diarrhea were described as two different diseases (32). We now know that these clinical manifestations could have been syndromes caused by Noroviruses (NoVs) in which the host’s individual characteristics (genetic and environmental) can affect the symptomatology of the disease (79). The first well documented epidemiological report of an outbreak, which was years later confirmed as a NoV infection, occurred at a school in Norwalk, Ohio in 1968. In two days, 116 of 232 children and adults attending the school manifested nausea and vomiting, and in fewer cases, diarrhea. The symptoms lasted 24 hrs and there was a secondary attack rate of 32% from contacts to the primary cases (214). A sample taken from a secondary case was used as inoculum for volunteer studies that provided material and the necessary information to identify an etiologic agent years later (79, 214).
1.1.1 Discovery

It was not until 1972 that the use of electron microscopy (EM) on a volunteer fecal specimen (designated 8FIIa) incubated with convalescent sera from a volunteer permitted the visualization of 27 nm viral particle aggregates (214). This was the first virus identified as a cause of gastroenteritis and it was quickly followed by the identification of other viruses such as rotavirus, adenovirus and astrovirus. The calicivirus strain that was in the 8FIIa sample was designated Norwalk virus. At that time a code was implemented to attain objectivity on the amount of virus-antibody interaction. The scale from 0 (no coating of the virus) to +4 (full coating and aggregation) is still used today.

1.1.2 Early volunteer studies

Several volunteer studies to identify the agent of nonbacterial gastroenteritis were performed in United States (U.S.), United Kingdom (U.K.) and Japan from the 50’s to the 70’s using stool filtrates. In every case results were inconclusive as only a percentage of the volunteers become infected (32). Furthermore, some volunteers who were resistant to the infection did not seroconvert and did not appear to have preexisting immunity to protect them. A clinical study demonstrated that 3 clinical outcomes were possible when volunteers were inoculated with the same fecal filtrate preparation: only vomiting; only diarrhea; or both symptoms (79). These studies also showed that Norwalk virus caused enteritis in the proximal small intestine and no lesions could be detected in the stomach and lower intestine (288, 380). Cross challenge studies demonstrated that some strains were antigenically related whereas others were not (468).
At that time, many questions remained unanswered until the 1990’s when molecular biology techniques offered new approaches and findings regarding the strains relatedness were confirmed by phylogenetic analysis (28).

1.1.3 Early experimental animal studies

Several animals models such as mice, guinea pigs, rabbits, kittens, calves, baboons, marmosets, Rhesus macaques, owl monkeys, patas monkeys, cebus monkeys and chimpanzees were evaluated in the 1970’s and 1980’s (136). Of these only chimpanzees shed virus although they were asymptomatic (469). Also 2 of 4 Rhesus macaques seroconverted and produced soft stools after oral inoculation with Norwalk virus (57). At that time molecular techniques were not available; therefore, viral detection was less sensitive. In addition, the role of histo blood-group antigens (HBGA) in the susceptibility and resistance to these viruses was unknown and the monkeys tested may have been of a resistant phenotype. Most of these experimental animal model studies were done using a single strain, Norwalk virus, the prototype of NoV genogroup I (GI) (136) (see section 1.5.5).

1.2 Etiology

1.2.1 Agent

Human enteric caliciviruses, both NoVs and Sapoviruses (SaVs), belong to the family Caliciviridae. These are small (27-40 nm in diameter) nonenveloped viral particles carrying a single-stranded, plus sense RNA genome (140). The SaVs can be
differentiated morphologically from other enteric viruses by having a characteristic surface with “calyx” structures giving the appearance of “Star of David” (Figure 1.1) (15, 141). Before genome classification was available, NoVs were called Small Round Structured Viruses (SRSV) (140, 236). Their shape is round but with no typical surface characteristics in contrast with SaVs (Figure 1.1).

1.2.2 Taxonomy

The taxonomy of this family includes four genera (Figure 1.2.) (134): a) the Vesivirus genus, which cause epithelial vesicles and reproductive failure in swine (VESV) and marine mammals (SMSV) (308), respiratory disease in domestic and wild cats caused by feline calicivirus (FCV) and enteric disease in caused dogs by canine calicivirus (CaCV) (90, 106); b) the Lagovirus genus that cause hemorrhagic disease in rabbits and hares (RHDV and EBHSV) (290, 299); and c) the NoVs and d) SaVs. These two latter names were approved by the International Committee on the Taxonomy of Viruses (ICTV) for the previously named “Norwalk-like viruses” and “Sapporo-like viruses” (284). Human enteric caliciviruses that cause gastroenteritis belong to these two genera. Also, strains in these two genera cause diarrhea in cattle, swine and mink (38, 39, 64, 65, 99, 148). There is a cultivable strain of mink calicivirus (MCV) that is morphologically similar to the non-cultivable mink strain that belongs to the SaV genus (MEC) and causes enteritis (section 1.5.1.8). The MCV in contrast does not cause gastroenteritis and is genetically closer to the vesivirus strains (150). In addition, there is an enteropathogenic bovine enteric caliciviruses (BEC), Nebraska strain (NB) that is still unclassified (388). Although NB has similar morphology and induces clinical
manifestations similar to bovine NoV GIII, its genome is organized into two open reading frames (ORF) as the SaV and Lagovirus genera (section 1.2.3). Amino acid sequence comparison for the partial ORF1 of NB (region encoding for the non-structural proteins) showed identities of only 14.1 to 22.6% with NoV, SaV, vesivirus, and lagovirus strains but, the conserved motifs of the non-structural proteins shared among caliciviruses were present. The NB capsid protein (VP1), also in ORF1, sequence identity was low compared with other caliciviruses (14.6 to 26.7%) and phylogenetically was closest to strains in the Lagovirus genus although NB clinical signs are enteric and not hemorrhagic (sections 1.5.1.2 and 1.5.1.6). Thus, this strain may represent a new genus within the Caliciviridae family as supported by the recent description of the Newbury-1 (NA-1) genome sequence characterization (326).

At present NoVs are classified into 5 genogroups (G) based on their capsid protein nucleotide sequence of ORF2 (Figure 1.2) (475). The GI is composed of human strains, GII includes human and porcine strains whereas GIII contains 2 bovine strains, Newbury Agent-2 (NA-2) and Jena (11, 475). The fourth (GIV) genogroup consists of a single human strain named alphatron (445) and the murine NoV (MNV-1) represents the fifth genogroup (GV) (475). Within those 5 genogroups at least 29 genoclusters are identified. SaVs are also classified into 5 genogroups according to their capsid region: GI, GII, GIV and GV comprising human strains and GIII consisting of the PEC/Cowden strain (96, 382). It was proposed that MEC belongs to a separate genogroup within the SaV genus (150). Both classification of NoV and SaV is subject to change as new strains from both human and animal origin are detected constantly (445, 449, 451). The capsid inter-genus nucleotide sequence differences (between NoV and SaV) range from 84-86%.
For SaV inter-genogroup, inter-genocluster and intra-cluster nucleotide differences are 49-55%, 19-25% and 1-5%, respectively, and for NoV, they are 47-56%, 24-37% and 1-4%, respectively (382). Classification of NoV is based on the whole capsid sequence because it is more meaningful to the antigenic relatedness of the virus and because of the existence of recombinants so that classifying by the RNA-dependant RNA polymerase (RdRp) sequence could give different trees (166). Also, conserved regions among distant strains such as within the RdRp could not differentiate genotypes whereas highly variable sequences in the genome including the P domain in the capsid gene or in the VP2 (small basic protein) could not separate genogroups or genus, respectively (218). The proposed nomenclature for NoVs and SaVs is: species infected/ virus genus/ virus name/ strain designation/ year of isolation/ country of isolation (15). The prototype strains of each cluster are usually referred to by their common name which usually corresponds to the location of the isolation.

1.2.3 Genome organization

It was not until 1990 that the genome sequence of Norwalk virus, a NoV from GI became available (194) and five years later the genome sequence of Manchester, a SaV was described (251). The genome organization is distinctive for NoVs and SaVs (Figure 1.3) (55). Both groups have a single-stranded RNA (ssRNA) genome of positive polarity of approximately 7.5 kb excluding a poly A tail. Noroviruses, as well as vesiviruses, have 3 ORFs whereas SaVs and lagoviruses have only two. The 5’untranslated region (UTR) has sequence diversity among genogroups (55). In NoVs ORF1 codes for six nonstructural proteins including the 2C-like helicase, 3C-like protease and 3D-like RdRp
terms used because of their similarity to poliovirus proteins. In NoVs this is a large polyprotein of approximately 1,750 amino acids depending on the strain, that is then cleaved by the viral protease in *cis* and *trans* (15). In NoVs, ORF 2 codes for the capsid protein of approximately 530 amino acids (56-62 KDa). This ORF overlaps at its 5’ end with the 3’ end of ORF1 by a few bases which vary in length, being 17 nucleotides for GI and 20 nucleotides for GII. A 2 kb RNA was detected in a stool sample of a Norwalk infected individual (202) suggesting that subgenomic RNA, like the ones observed in FCV and RHDV (290, 307), exist in this genus. In the murine MNV-1 infection of RAW cells (a macrophage tumor cell line from mice, commercialized by ATCC); a similar 2.3 kb RNA was detected (464) as well as in the PEC/Cowden strain of SaV (50). This subgenomic RNA codes for the capsid protein and the ORF3 basic protein (VP2). In the *Lagovirus* genus the subgenomic RNA is encapsidated together or separately from the full-length genome (290). The RHDV viral particles containing genomic, subgenomic or both types of RNA had different densities when purified through a sucrose gradient. It is unknown whether NoV or SaV pack subgenomic RNA into the viral particles but the fact that an RNA of similar size was detected in human stools suggest that this nucleic acid, that would be quickly degraded in the gut environment by RNAses, must be protected by the capsid.

The SaVs have an ORF1 which codes for both the nonstructural and the capsid protein whereas ORF2 codes for a small basic protein. An additional small ORF was predicted overlapping the capsid gene but in a +1 reading frame (192). This region is conserved in other SaVs such as Manchester and Plymouth virus but is absent in London strain suggesting it may not have an essential biological function (55).
1.2.4 Structural and non-structural proteins

The ORF1 of NoVs encodes a polyprotein of approximately 1,800 amino acids and 200 kDa that is autocatalytically processed by the viral protease into at least 6 nonstructural proteins (p48, p41, p20, VPg, protease, RdRp) (26). In some cases their functions have been difficult to deduce because of the lack of a cell culture system for the enteric strains. Subsequently the studies have been performed with the FCV and RHDV surrogates that do grow in cell culture and also with the use of *in vitro* replicons for the NoV and SaV (26, 250).

The nonstructural proteins terms, order and presumed function from the N-terminal end of the viral proteins are the following:

**p48** The N-terminal protein, also called p48 shows no significant sequence similarity to any viral or cellular proteins, and its function is unknown (88). This protein displays a vesicular localization pattern in transfected cells when fused to a fluorescent reporter. A C-terminus of p48 transmembrane domain would redirect the localization of the reporter gene to a fluorescent pattern consistent with the Golgi apparatus, but was not necessary for the observed vesicular localization pattern (88). The p48 might disrupt intracellular protein trafficking. Using a yeast two-hybrid screen, the *N*-ethylmaleimide sensitive factor attachment protein receptor (SNARE) vesicle-associated membrane protein-associated (VAP-A) was identified as a binding partner of p48 and showed that expression of a cell surface glycoprotein was inhibited when cells coexpressed p48. The VAP-A is a known regulator of vesicle transporting.
**p41** The p41 shares sequence motifs with protein 2C of picornaviruses and superfamily 3 helicases. The p41 of the Southampton strain was cloned and expressed in bacteria and after purification it exhibited nucleoside triphosphate (NTP)-binding and NTP hydrolysis activities (347). The NTPase activity was not stimulated by single-stranded nucleic acids. The p41 had no detectable helicase activity. Its specific function during viral replication is unknown but because its NTPase activity is inhibited by homopolymeric RNA this protein might be necessary during early stages of viral replication and not in later steps. Protein sequence comparisons between the consensus sequences of NoVs p41 and enterovirus protein 2C revealed regions of high similarity. According to secondary structure prediction, the conserved regions were located within a putative central domain of alpha helices and beta strands.

**p20** or p22 depending on the genogroup has an unknown function (26).

**VPg** The virus protein genome linked (VPg) is a protein of 16 kDa that is covalently linked to the 5' end of the viral genome (40). The lack of a cap structure typical of eukaryotic mRNA and absence of an internal ribosomal entry site (IRES) suggests that VPg may function in translation initiation for calicivirus RNA through a protein-protein interaction with the host’s translation machinery (66). Norwalk virus VPg binds to cellular translation initiation factors. The eIF3d subunit of eIF3 was identified as a binding partner of VPg by yeast two-hybrid analysis and also by using purified mammalian eIF3. The VPg inhibited translation of all reporter RNAs (capped reporter RNA, an RNA containing an encephalomyocarditis virus (EMCV) IRES or an RNA with
a cricket paralysis virus IRES) in a dose-dependent manner when added to cell-free translation reactions. The N-terminal sequencing of the 15.5-kDa protein shows that amino acids 961-980 of the FCV ORF1 polyprotein encode the FCV VPg (406). *In vitro* studies using the FCV system shows that VPg removal resulted in loss of infectivity and decreased translation but this could be countered by capping a full-length cDNA clone (174).

**Protease** The protease forms part of the polyprotein encoded in ORF1 and it is responsible for the cleavage of the different protein intermediates and individual proteins in *cis* and with less efficiency in *trans* (26, 35, 386). This enzyme of 19 kDa is a member of the cysteine proteases as determined by site directed mutagenesis of its catalytic cysteine residue, its active site forming a catalytic dyad without a carboxylate. This anion, R-CO$_2^-$, plays an important role in folding and function of proteases from other viruses (165, 403). The NoV protease displays sensitivities to cysteine and serine protease inhibitors similar to poliovirus 3C protease (35, 402).

A proteolytic cleavage map was determined by cloning the ORF1 of a NoV GII/4 into an expression vector. Five cleavages at positions (Q(330)/G(331), Q(696)/G(697), E(875)/G(876), E(1008)/A(1009), and E(1189)/G(1190) gave the following final products: N-terminal protein; nucleoside triphosphatase; 20 kDa protein (p20); VPg; protease (Pro); RdRp (or Pol), respectively. There were also active intermediate products, p20-VPg (analogous to the 3AB of the picornaviruses) and ProPol (analogous to the 3CD of the picornaviruses) a bifunctional enzyme during virus replication (25). Also, less stable products: p20VPgProPol, p20VPgPro, and VPgPro were detected (26). The
optimal pH and temperature of the protease of Chiba strain for proteolytic activity is 8.6 at 37°C, respectively. High concentrations of monovalent cations such as Na\(^+\) and K\(^+\) inhibit its activity as well as divalent, Hg\(^{2+}\) and Zn\(^{2+}\) whereas Mg\(^{2+}\) and Ca\(^{2+}\) have no effect (402).

The NoV protease may also play another role in viral replication by inhibiting cellular translation as demonstrated for FCV and a strain of NoV GII/4 in a similar fashion to poliovirus protease (235). The proteases of these two were found to cleave poly(A)-binding protein (PABP) by separating the C-terminal domain (that binds translation factors eIF4B and eRF3) from the N-terminal RNA-binding domain. Analysis of FCV-infected feline kidney cells showed that the levels of *de novo* cellular protein synthesis decreased over time as virus-specific proteins accumulated, and cleavage of PABP occurred in virus-infected cells (235).

**RNA-dependent RNA polymerase** The RdRp has a molecular weight of 57 kDa. It synthesizes both genomic and subgenomic viral RNA in a primer and poly(A) independent way as demonstrated by using a recombinant baculovirus system to express an enzymatically active RdRp protein from the Norwalk virus genome (105). The YGDD sequence motif is highly conserved among all caliciviruses because it is involved in the catalytic activity of the enzyme (440). Therefore, this region is usually targeted for primers meant to have wide detection properties (197).
In vitro mapping studies of the MD145 GII/4 NoV ORF1 polyprotein identified two stable cleavage products containing the viral RdRp domains: ProPol (a precursor comprised of both the protease and RdRp) and Pol (the mature RdRp) both with RNA polymerase activity (25).

**VP1** This protein of approximately 550 amino acids and 58-64 kDa is encoded in ORF2 of NoVs and ORF1 of SaVs (55) and it is the major structural protein. Expression of the capsid protein in different systems such as baculovirus (201), bacterial (423) or mammalian cells (424), results in self-assembly into empty virus-like particles (VLPs). This assembly into VLPs has been very useful as a consistent source of pure viral antigen since human NoVs do not grow in cell culture and are shed in feces in low titers (138). The availability of NoV VLPs has helped the development of better diagnostic tools and have served in the study of the structure of viral particles. Also, the recombinant viral particles are antigenically similar to the native virus, thus having a potential role as vaccines, use in epidemiology studies and to study virus-host receptor interaction. Early studies showed that morphologically identical NoVs by EM belong to at least two genetically distinct groups (140) and that different antigenic types of NoVs exist (141). Three different domains have been described in VP1 of Norwalk virus: from the N-terminal to the amino-terminal: the shell domain (S) coding for amino acids 1 to 225 and a protruding domain (P) that is then subdivided into a central stem domain (P1A, amino acids 226 to 278), a globular domain (P2, amino acids 279 to 405) that is bilobed, and another section of the P1 stem (P1B, amino acids 406 to 520) (Figure 1.4) (353). The P2 contains a hypervariable region that could be surface-exposed and that contains the
NoVs antigenic determinants (141, 352) and may be subject to an immunity-driven mechanism for viral evolution (313). The three-dimensional structure of the baculovirus expressed Norwalk virus capsid has shown that the empty particle is 38 nm in diameter, exhibits T=3 icosahedral symmetry and is composed of 180 capsid proteins forming dimers. Although there are similarities with the structures of T=3 ssRNA viruses, distinctive structural architecture such as arch-like capsomeres formed by dimers of the capsid protein and large hollows at the icosahedral axes differentiates this family (Figures 1.4) (353). Smaller particles of approximately 23 nm in diameter have also been observed, having a different symmetry (T=1) and only 60 copies of VP1 (456). Structural studies of VLPs of caliciviruses from different clusters (GI/4) or genera (SaV GI/2 and Vesivirus San Miguel sea lion virus, SMSV) showed that they all shared the T=3 icosahedral symmetry (54). Also, a smaller form of the capsid protein of 30 kDa designated as soluble protein has been detected in feces of infected humans (143). An in vitro digestion of recombinant Norwalk virus VLPs with trypsin showed that the 30kDa protein in stool is produced by specific cleavage of the Norwalk virus capsid protein in vivo and that the cleavage occurs at amino acid residue 227. An estimated 50% of the viral antigen excreted in stools is soluble antigen (167). Although the biological importance of this cleaved product is unknown, the presence of a large amount of soluble capsid protein may influence the host’s immune response, especially if the cleaved product presents the most immunodominant epitopes (167) or affects the pathogenicity of Norwalk virus infections (168). The expression of the recombinant VP1 of SaVs differs from NoVs in that the capsid protein is encoded in ORF1. An upstream sequence of 73 nucleotides was necessary for production of the capsid protein in a baculovirus
expression system (204). Therefore SaV and RHDV may have similar expression
requirements that differ from the NoV and vesivirus may be due to their distinct genome
organization (163).

**VP2** The small basic protein coded by ORF3 in NoVs and ORF2 in SaVs is
poorly conserved among the *Caliciviridae* family (141) varying in size (22-29 kDa for
NoVs and 29 kDa for SaVs) and amino acid composition, so it might have different
function in each genus (46, 385). It is expressed from both genomic and subgenomic
RNA (173). Several roles have been suggested for this protein (119). It regulates the
expression of VP1 and adds stability to the assembled virus particles (29). In FCV this
protein is 8.5 kDa (406) or 12.2 kDa and 106 amino acids (405). The difference between
the calculated and the apparent masses in SDS-PAGE is not due to proteolytic processing
of the protein (406). Others reported a mass of 10 kDa for the ORF3 product of FCV. In
the latter study the protein could be detected in FCV-infected cells but not in the purified
particles. No neutralization activity against FCV was detected testing VP2 antisera
prepared in mice. Thus this protein may be in the interior of the particle or it may
function as a non-structural protein for FCV (433). The FCV VP2 may interact with the
capsid and the genomic RNA being responsible for its encapsidation (309). When the
ORF3 was deleted from a FCV infectious clone it was lethal to the virus whereas small
deletions and stop codons were tolerated for the genome replication but resulted in non
infectious particles. However, infectious virus could be recovered after adding VP2 in
*trans* to rescue the defective clone (405).
It was determined that VP2 is present in the viral particle in 1 or 2 copies by radioactivity incorporation into the viral protein during \textit{in vivo} labeling (406). In RHDV it is a minor component of the mature virion participating in particle assembly (462).

\textbf{1.2.5 Biological characteristics}

Some physicochemical characteristics of NoVs are difficult to evaluate in the absence of a cell culture system. Several surrogates have been used (FCV, CaCV, bacteriophages) (67, 83) but the information they provide is incomplete. For example, FCV and CaCV inactivation was correlated to reduction in PCR units for FCV, CaCV and NoV, but detection of viral RNA underestimated reduction in virus infectivity. The NoVs are resistant to temperatures of 60°C for 30 minutes and to low pH (230). They are also resistant to commonly used levels of chlorination (0.5-1 mg/l), but they are inactivated at 100°C and by high concentrations of chlorine (>2 mg/l). The NoVs are non-enveloped viruses so it is not surprising that inactivation with 70% ethanol is inefficient with only 1 log$_{10}$ reduction after 30 minutes (83). The human NoVs were more resistant than the animal vesiviruses to low (pH2) and high (pH12) pH, maybe due to adaptation to the stomach and gut environment. Viral RNA could be detected from NoV, FCV and CaCV samples after 3 minutes at 100°C, although FCV and CaCV had lost their infectivity this could not be evaluated for the human NoV. Both animal caliciviruses showed a dose dependent inactivation by non-ionizing ultraviolet (UV)-B irradiation with a 3 log$_{10}$ reduction at 34 mJ/cm$^2$. This is a much lower value than that described (120-200 mJ/cm$^2$) for the same two viruses by others (69). Ionizing radiation (gamma) in contrast to UV was dependent on protein concentration. Both types of radiation were different for
caliciviruses than for MS2 bacteriophage. The NoVs have a buoyant density of 1.33-1.41 g/cm$^3$ in CsCl whereas SaVs have densities of 1.37-1.38 g/cm$^3$ (136, 428). Norwalk virus was still infectious to human volunteers after the stool filtrate was maintained at pH 2.7 for 3 hrs at room temperature, treated with 20% ether for 18 hrs at 4°C or maintained at 60°C for 30 minutes (78).

1.3 Diagnostics

There is not a single gold standard method to detect caliciviruses. No diagnostic method by itself is reliable enough and usually combinations of them are used to confirm an outbreak (15).

1.3.1 Reverse transcriptase polymerase chain reaction (RT-PCR) and reaction inhibitors

The RT-PCR technique is highly sensitive and applicable to epidemiological studies but the presence of reaction inhibitors in stools, vomit, serum and environmental sample and the high genetic diversity of human NoVs and SaVs result in large numbers of false negative samples (15). Many approaches were taken to detect more strains and reduce the number of false negatives by RT-PCR: a) nucleic acid extraction methods that would eliminate inhibitors; b) the use of an internal standard reaction control to detect the presence of reaction inhibitors; c) and broader reactive primers that could detect as many strains from different genogroups and from both NoV and SaV genera.
a) **RNA extraction methods.** In the case of RNA extraction methods for calicivirus from stool samples, many protocols have been tried (228) and compared including dialysis, heating, phenol-chloroform extraction and oligo-dt cellulose chromatography (201). None of these methods were very efficient in removing inhibitors. The precipitation with polyethylene glycol (PEG) and the addition of the detergent cetyltrimethylammonium bromide (CTAB) to the extraction increases the quality of the RNA. Another study evaluated Chelex-100 (a metal chelating agent) and Shephadex G200 column chromatography, guanidinium thiocyanate (GTC)/silica method and PEG precipitation followed by phenol-chloroform extraction with the use of CTAB, with regards to their extraction efficiencies and their ability to remove inhibitors. From these, the column chromatography method was the most sensitive but was not efficient in removing the inhibitors (17 and 56% of inhibited samples respectively). The methods using PEG precipitation followed by phenol-chloroform extraction with the use of CTAB were less sensitive, detecting viral RNA in sample dilutions of 1:10 and inhibition was observed in 19% of the samples whereas the column chromatography methods detected viral RNA in $10^{-4}$ sample dilutions. In conclusion the GTC/silica was sensitive and removed inhibitors from all samples (157). This was further supported by a “universal” extraction method for all gastroenteric viral agents (NoVs, SaVs, astrovirus, rotavirus and adenovirus): GTC/silica followed by phenol-chloroform-isoamyl alcohol were the best extraction methods (357). An additional method that was both fast and simple was evaluated for Norwalk virus, consisting of trichlorotrifluoroethane (Freon) extraction of 10% sample dilutions followed by centrifugation, then a 100-fold dilution was heated at 95°C for 5 minutes, chilled on ice and immediately tested by RT-PCR (384). The
efficiency of this procedure was similar to that of conventional methods. Heat not only
denatured the capsid protein, but also released the RNA and inactivated some inhibitors.
The advantages were simplicity and low cost. There is a nucleic acid isolation benchtop
instrument (MagNa Pure, Roche) that is capable of extracting 32 samples in parallel
(379). The software guides the robot in every step which includes, lysis, proteinase K and
nucleic acids bound to glass particles and washes. This equipment could be very useful in
diagnostic and research labs where large numbers of samples need to be tested routinely.
Efficiency in isolation and subsequent detection with this instrument was comparable to
traditional methods but was faster.

b) **Internal control.** Different ways of avoiding and detecting PCR reaction
inhibitors are: sample dilution; addition of a target nucleic acid to the reaction; the use of
a second pair of primers to detect another sequence that is present in the sample; and the
use of an internal standard control (15). An internal control for Norwalk virus with a
deletion of 123 base pairs of the product of primers p35-p36 was designed (384).
Differential size and 2 different hybridization probes unequivocally permitted
identification of the product and the internal control. The engineering of the internal
control involved cloning of the target sequence into a vector with specific primers that
have complementary sequences with alternate internal deletions. Internal controls are
essential when testing water samples where reaction inhibitors are concentrated together
with viruses during sample processing (339).
Competitive internal controls for viruses commonly found in water (Norwalk virus, poliovirus, hepatitis A and rotavirus) were developed by RT-PCR, restriction enzyme, ligation, cloning and \textit{in vitro} transcription of the recombinant (virus/internal control) RNA (339).

c) Primers. When designing primers for human enteric caliciviruses for RT-PCR, the application must be taken into account. Usually, for epidemiological screening of a large number of samples with highly divergent strains, a broadly reactive primer pair is desired, even if this causes decreased sensitivity. For example, some of the approaches taken to detect as many different human enteric caliciviruses as possible have been the design of degenerate primers for both GI and II NoVs and some SaVs (241); multiplex reactions (using a unique primer for the reverse transcription reaction and 4 primers for GI and 1 for GII NoVs) (9); and the design of a pair in the most conserved region of the viral genome (the RdRp gene) (197). A broadly reactive primer pair P289/290 was designed to detect most NoVs and many SaVs including some PEC and BEC strains (149, 389). However, this primer pair was shown to cross-react with sequences in other viruses (rotavirus) (261). When a particular strain needs to be detected, these approaches may not be sensitive enough and specific primers designed to the desired strain need to be selected (274, 375). Many primers were described for detecting certain genera or genogroups in particular (Table 1.1). Because the RdRp is the most conserved region of the viral genome, other regions such as the helicase, the capsid and the small basic protein have been targeted for amplification so as to increase the sequence information to differentiate strains. Primers in these regions are less broadly reactive. Degenerate
genogroup-specific primer sets targeting the C-terminal region of the capsid gene (designated region D) could detect 95 of 100 known positive samples and the sequence information they provided was useful to differentiate among genocusters (444). Another primer pair targeting the capsid region could differentiate GI and GII and had increased sensitivity by nested PCR when compared to other existing primers for NoVs (227). Primers targeting the capsid region give information that is useful for classifying isolates (142). However, the recent description of recombinant strains shows the need for amplifying more than one region in the genome before classifying a new strain (274).

Another consideration is the sensitivity of the primer pairs. Some can only detect the virus when present in large amounts whereas others are independent of the number of viral particles, the exact reason for this is not clear but it is probably related to the degree of conservation and whether the target area presents secondary structure. Because of primer homology, the more specific the more sensitivity (15). Increased sensitivity can be obtained by reducing the annealing temperature but this is inversely correlated to specificity and several nonspecific bands, including some of the expected size may appear. To overcome problems in variation of viral detection among laboratories due to different RT-PCR techniques; several laboratories were involved in the evaluation, comparison and optimization of primers (441, 446). For example a primer pair with a sensitivity of 25 virus copies was reported in the detection of MNV-1 (179). The use of nested PCR also increases sensitivity (131), giving a detection rate of 97% (331), but it is difficult to avoid carryover contamination and it increases time and costs. The use of more efficient DNA polymerases in the PCR and the addition of thermolabile UNG (uracil-N-glycolase) that prevents carryover contamination have been useful for
increasing sensitivity and preventing false positive results (131). Another method described a double amplification performed with the same primer pair used ten times more concentrated in the second amplification and with longer annealing time (68). This so-called RT-booster PCR was performed with primer pair JV12 and 13 for detection of NoV in shellfish. Therefore, the 2-log increase in viral detection may be useful in detecting NoV in samples that may have very few viral particles.

Multiplex RT-PCR have been developed to detect NoVs from different genogroups (335) together with other enteric viruses such as adenovirus, rotavirus (330) astrovirus and SaV (368). The advantage of a multiplex reaction is the cost and the reduction in time when determining the agent causing an outbreak.

A non RT-PCR method known as nucleic acid sequence-based amplification (NASBA), previously established for detection of several other viruses, was applied to NoV detection (144). This technique could detect 10,000 RT-PCR detectable units. It is based on an isothermal reaction that takes 90 minutes and utilizes 2 target-specific primers: the reverse primer has a tail containing the T7 promoter sequence and the forward primer contains an electrochemiluminescent tail that is used for detection. The 3 enzymes used are retrotranscriptase (RT), T7 RNA polymerase and RNAse H. Another study showed that RT-PCR and NASBA detection could be complementary (296). When comparing a panel of 17 NoV known-positive samples NASBA detected 13, the remaining were 3 GII and 1 GI strains. However, when testing outbreak samples NASBA detected 13 samples that were negative by RT-PCR. The use of this technique applied to RT-PCR products can result in 1-log$_{10}$ enhancement in viral detection (188). The NASBA can also detect several enteric viruses in a multiplex format (189).
1.3.2 Real-Time RT-PCR

In the last three years real-time RT-PCR has been applied to the study of caliciviruses. The advantages of using light cycling technology are the speed, sensitivity and specificity which have great importance when dealing with an outbreak. The ability to determine the product’s identity by their melting points in the melting curve alleviates the need for gel electrophoresis and sequencing saving time and costs. Both SYBR Green (30, 239, 292, 300, 334, 361, 362, 379) and Taqman (178, 210, 255, 314, 335) biochemistries as well as 1 step (178, 255, 361, 362, 379) or 2 step (30, 210, 239, 292, 314, 334, 335) RT-PCR have been used and compared. Each technique presents advantages and disadvantages for each given purpose. For example, Beuret (2004) designed a multiplex real-time RT-PCR for detection of NoVs of GI and GII, astroviruses and enteroviruses using a 2 step SYBR Green reaction. In this same work the use of Taqman probes showed lower sensitivity. Two step reactions can increase the sensitivity up to 10,000 times when compared to traditional RT-PCR, detecting from 5 to $5 \times 10^6$ copies of RNA per reaction or an estimate of 25,000 viral genomes per gram of stool (334) or 100 times greater sensitivity than 1 step reactions (335). In some cases the standard curve was created with RNA transcribed from a plasmid (334) in opposition to other work in which cDNA was used (178, 210, 240, 335) or serial dilutions of a known positive sample (255, 361, 362). Myrmel et al (2004) performed real-time PCR (after RT-PCR) in order to quantitate virus load in shellfish although this increased the time and costs.
1.3.3 Antibody and antigen enzyme-linked immunosorbent assay (ELISA)

The cloning of the capsid gene and expression in insect cells by a baculovirus system, bacterial (473) or mammalian cells (349, 423) were breakthroughs in the preparation of diagnostic reagents. When expressed the capsid protein self-assembles into VLPs (201). The hyperimmune antiserum from animals inoculated with VLPs improved the antigen ELISAs and the VLPs were also used to replace purified virus particles as they are similar in morphology and antigenicity and can be produced in high concentration and purity for antibody detection (138). The ELISA has replaced radioimmune assays (RIA) because of the increased stability of the reagents (months for ELISA and only days or few weeks for RIA) and to avoid the use of radioisotopes (176). Many VLPs have been engineered from both NoV and SaV (137, 156, 198, 204, 205) (Table 1.2). Some antigen ELISAs have a sensitivity of $10^5-10^6$ particles, and are specific to human enteric caliciviruses as they do not detect other enteric viruses such as rotavirus, adenovirus, astrovirus, enterovirus and hepatitis A virus (191, 200). Some antigen ELISA may be cluster-specific and may allow a quick assignment of strains to different genotypes according to their reactivities (213). The polyclonal antisera produced to each VLP are specific for detecting homologous or closely related VLPs but not for reacting to most heterologous particles (124, 191). Thus, their application is limited in field studies unless used as pools but, they are very useful in volunteer studies. Also, the immunization of animals with multiple VLPs partly overcame this problem (196). Antigen ELISAs with monoclonal antibodies (MAbs) have also proven to be highly sensitive and specific (175). A SaV PEC VLP ELISA was developed to detect antibodies against SaV PEC Cowden strain (152), and with the development of VLPs
and antibodies to these from both NoV GIII and NB-like strains, ELISAs for both BEC groups are now available and have shown no cross-reactivity with human NoV VLPs from GI and GII (162). However, another study reported cross-reactivity between the VLPs from GIII NoV with the GI NoV of human origin (459).

Antibody ELISAs using VLPs as antigens were developed to detect total, immunoglobulin (Ig) class and subclass specificity in serum as well as strain-specific (Norwalk virus) fecal IgA antibodies (201, 325, 434). A NoV antibody ELISA detected 98% of infections whereas the antigen ELISA detected 88% in an experimental study (124). Homologous seroresponses (316) and in a few cases heterologous (434) responses were reported. Antibody ELISA using the VLPs are quite specific, sensitive, and efficient for detecting specific antibodies, and these assays have been used in several large-scale seroepidemiologic studies (74, 127, 203, 337). The Norwalk virus-VLP ELISA detects broadly reactive antibody responses to NoVs both in GI (Norwalk virus) and GII (Hawaii, Snow Mountain virus) infected volunteers, although the maximum response was observed in volunteers challenged with the homologous Norwalk virus (124, 434). For this reason, presumably due to pre-existing human enteric calicivirus antibodies, it is difficult to identify the antigenic type of the infecting strain by seroconversion as measured by ELISA (316). An antibody ELISA was used to detect IgA and IgG in saliva to compare to serum antibodies in 38 volunteers inoculated with Norwalk virus (see section 1.6.1) (295). The combined detection of both salivary IgA and IgG antibodies was sensitive (100%) and specific (95%) in detecting the 18 individuals that became infected.

Commercial ELISAs to detect NoV in feces are already available (41, 360). The IDEIA™ (ELISA; Dako Cytomation, Ely, U.K.) can detect NoV antigen in fecal samples
and determine the NoV genogroup and does not cross-react with at least one human SaV strain and the bovine NA-2 strain. When compared with RT-PCR, the ELISA had a sensitivity and specificity of 55.5 and 98.3%, respectively, whereas EM has 23.9 and 99.2%, respectively. This commercial ELISA was also compared to a commercially available Japanese antigen ELISA kit, SRSV(II)-AD (Denka Seiken Co. Ltd., Tokyo, Japan) (41). The sensitivities and specificities of the two kits were tested with a panel of NoV- and SaV-positive stool samples from both NoV genogroups from several clusters and samples containing other viruses were used as controls. The Japanese kit (Denka) had a sensitivity of more than 70% for most of the clusters but the specificity was low (69%). In contrast the Dako kit had lower sensitivity (<30% for 6 GII subgroups) but a high specificity of 100%. Another 4 clusters (GII/2, GII/5, GII/6, and GII/n) could not be detected by the Dako kit. The Dako kit could discriminate between the GI and GII antigens of NoV, the Denka kit cross-reacted with samples containing GI and GII and also reacted with samples containing SaVs. In addition, the Denka kit reacted with 13% of the negative samples showing the high sensitivity is accompanied by low specificity.

1.3.4 Immune electron microscopy

The EM technique is useful for screening for unknown viruses or identifying mixed infections but because it is laborious, time consuming and requires experience and expensive equipment and a high viral load, it is not used routinely for detection of NoVs and SaVs (15). The EM has a detection limit baseline of $10^6$ virions per ml of stool (77). It is difficult to observe NoV particles because they do not posses a characteristic shape as SaV do (see section 1.1). Therefore, the use of specific antibodies to coat and
aggregate the viral particles in what is known as immuno EM (IEM) increases the detection rate. The IEM procedure involves low-speed centrifugation to clarify a 10% stool suspension, virus concentration by ultracentrifugation or ammonium sulfate precipitation, incubation with serum (reference serum or convalescent) and precipitation of immune complexes by ultracentrifugation. Phosphotungstic acid, uranyl acetate or ammonium molybdate are used to negatively stain viral particles (Figure 1.1) (77). There is a scale from 0 (no coating of the virus) to +4 (full coating and aggregation) to describe the amount of virus-antibody interaction (214). The IEM could detect only 50% of individuals clinically ill in a Norwalk virus infection volunteer study (427). In Japan 30% of sporadic cases and 60% of outbreaks were confirmed by IEM (324). To improve the detection rate, SPIEM (solid-phase immune microscopy) was developed where specific Igs were used to coat the grids and capture the virions (246). The use of protein A improves the system by attaching the Igs via the Fc portion making the antigen binding site more available (15). An automated system consisting of a Windows™ operated TEM, a CCD camera and an image processor could detect 95% of the virus-like particles that were detected by an operator on semipurified stools (436). NoV and SaV were stained with 2% uranyl acetate (pH4) and the image parameter was set at 30±6 nm for particle diameter. Although appealing the detection rate of the automated system, when compared to NoV-positive samples detected by the conventional IEM, was only of 56-78%.
1.3.5 Microplate hybridization and heteroduplex motility assay (HMA)

Positive samples by RT-PCR need to be confirmed because false positives also occur (261). Sequencing is an expensive and slow method, with the advantage that it provides more information, depending on the target region and the product length. Although the use of real-time RT-PCR has decreased the need for sequencing amplification products and the availability of sequencing facilities are more extensive than before, the development of quick and economic means of corroborating product specificity has been of great use in the laboratory. The PCR and RT-PCR may produce non specific bands that when observed in an agarose gel may or not differ in size from the expected product. A fast and inexpensive method that can be used to study outbreaks and large-scale epidemiological studies is hybridization, used in variable forms including Southern blot, dot blot, liquid and microplate hybridizations (10, 27, 37, 104, 187, 226, 282, 445, 450). Due to the genetic diversity of NoVs and SaVs, broadly reactive probes can not be used, but multiple probes to both genus and several genogroups have been tested successfully (27, 37, 282, 445, 450). Post amplification hybridization in both liquid (27) or microplate format (37, 282, 450) are based on the same principle of denaturation/renaturation although the methods vary in whether the double stranded DNA is denatured by heat or chemically (pH) and in the order and labeling of the products and probes. The same principle applies in the designated reverse line blot hybridization, based on the nucleotide divergence of a region of the RdRp gene which can be used to classify NoVs into genotypes (445). Amplicons were hybridized to 18 different membrane-bound oligonucleotides that were able to discriminate among 13 NoVs genotypes. By this method 94% (124/132) positive stool samples from 34 outbreaks and 20 sporadic cases of
gastroenteritis were genotyped and later confirmed by phylogenetic analysis. Phylogenetic analysis of the complete and partial ORF2 (capsid gene) sequences of the remaining 6% (8 strains from 3 outbreaks) that could not be genotyped revealed the existence of one novel genotype (Alphatron) and one potentially novel genotype (Amsterdam).

Another rapid (7 hrs) and economical technique called heteroduplex motility assay (HMA) was developed for amplicon identification without sequencing (281). The principle of this assay is based on mixing equal amounts of amplicon from the tested and reference strains, heating to denature and cooling to anneal the mixtures which are then run on an acrylamide gel. The visualization of heteroduplexes indicates that the strains are different whereas absence of these determines the strains identity. Sequences that are 90% identical can be identified by this technique as demonstrated by 130 outbreaks genotyped by HMA and sequence data correlated perfectly. The use of HMA was also useful in detecting the presence of multiple strains in environmental samples (130). Different HMA profiles when using amplicons from different representative strains showed the extent of NoV diversity within a sewage sample.

1.3.6 Magnetic beads

The presence of RT-PCR inhibitors occurs with very high frequency when working with fecal samples in studies of gastroenteric viruses. In the case of NoVs and SaVs, the impact of RT-PCR inhibitors on viral detection is even greater due to the characteristic low shedding of these viruses after the onset of disease (15). This has prompted the search for methods to overcome RT-PCR inhibitors such as
immunomagnetic capture assays (117). Paramagnetic beads coated with goat anti rabbit IgG were coupled to a hyperimmune rabbit polyclonal antibody to Norwalk virus VLPs, washed in a magnetic separator and blocked with 1% bovine serum albumin. The prepared magnetic beads were then incubated with 1:10 dilutions of the fecal samples for an hour, then washed and incubated at 95°C for 5 minutes to release the viral RNA, chilled on ice, centrifuged to pellet the beads and the supernatant was used in the RT-PCR. By this method the number of inhibited samples was reduced and the virus was concentrated. The drawback of this method is that only strains that are antigenically close to Norwalk virus would be captured. This problem could be overcome by using a mixture of antibodies with broad reactivities (472). The detection of a highly conserved epitope among NoVs could also solve this problem (See section 1.3.7). A similar approach involves the use of magnetic beads coated with streptavidin coupled to biotinylated HBGA which are known ligands of NoVs (170).

**1.3.7 Monoclonal antibodies (MAbs)**

At present there is an extensive panel of monoclonal antibodies to GI and GII NoVs (Table 1.3). They were first obtained for Norwalk virus and the antigenic map of this strain has been characterized (167). Some epitopes map to the C-terminal half of the capsid protein as determined by their recognition of the 32 kDa cleaved product as well as the complete 58 kDa capsid protein. Some MAbs recognize continuous epitopes in the C-terminal (detecting both denatured and non-denatured protein) whereas others react against discontinuous epitopes in either the N-terminal or the C-terminal (reacting only with non-denatured protein) (167). All of these MAbs aggregated Norwalk virus VLPs
but it is not possible to predict if these MAbs will have neutralizing activity without having a cell culture system available. A common epitope to GI NoV was identified by NV3901 MAb (159). Another research group obtained 17 additional MAbs to GI NoVs, 16 of which recognized continuous epitopes and only one recognized a conformational epitope (474). Two MAbs to GII NoV were broadly reactive even detecting the C-terminal of GI NoV capsid proteins (474). These two broadly reactive MAbs were mapped to recognize a 11 amino acid fragment in the N-terminal of the capsid protein, a region highly conserved among GI and GII NoVs (472). This research group suggested that the N-terminal of the capsid protein is more immunodominant (474) in contrast to what had been described by others where the C-terminal region contained more immunodominant epitopes (167). Another broadly reactive MAb mapped epitopes on the NoV capsid protein for both a GI-cross-reactive MAb and a GII-cross-reactive MAb by use of NoV deletion and point mutants (338). The epitopes for both MAbs mapped to the C-terminal P1 subdomain of the capsid protein. Recognition of a conformational epitope explains the MAbs’ genogroup specificity. Strain, genogroup specific (GI and GII) and genus specific (NoV and SaV) MAbs were produced by orally immunizing mice with either a single type of recombinant Norwalk virus (rNV), rKashiwa 47 virus, rSnow Mountain agent, or rSapporo virus VLP or with mixtures of two types of VLPs from different genogroups (225). Twenty MAbs were obtained as mouse ascites and characterized according to their ELISA and Western blot cross-reactivity patterns to VLPs. Five groups of MAbs reacted by both Western blot and ELISA and were classified as A) common cross-reactive MAbs for four GI and six GII NoV VLPs, B) NoV GI specific MAbs, C) NoV GII-specific MAbs, D and E) were strain-specific MAbs and
F) was one MAb that reacted only by ELISA. The group A MAbs, which showed broad cross-reactivity with VLPs of both NoV genogroups, were obtained from mice immunized orally with a single type of VLP (either rNV or rKAV). The only 2 MAbs available for SaVs, were obtained from mice immunized with SaV VLPs and reacted only with SaV but not with any NoV VLP.

The MAbs may be an important tool in the absence of a cell culture system for NoVs to define functional domains in the capsid that may be involved in virus-receptor interactions (254). Two MAbs to Norwalk virus (GI/1) and Snow Mountain virus (GII/2) VLPs that blocked binding of recombinant VLPs to Caco-2 intestinal cells and inhibited hemagglutination, were identified. Anti-Norwalk VLP MAb 54.6 and anti-Snow Mountain VLP MAb 61.21 recognized epitopes located in the protruding P2 domain. The epitope recognized by MAb 61.21 contained an amino acid motif IDPWI that is completely conserved among NoV strains across genogroups, including strains isolated from swine, bovine and murine species. Another MAb derived using Jena VLPs denominated CM39 reacted with both Jena and NA-2 which show little cross-reactivity by ELISA using polyclonal antibodies and also with human NoV GII/3 (327).

Monoclonal antibodies will be very useful in developing new diagnostic tests that can be efficient in detecting a wide variety of strains in the field and differentiating them (225). Some are already part of commercial diagnostic tests (338).

1.3.8 Hemagglutination and hemagglutination inhibition

Norwalk virus VLPs agglutinate RBC of humans and chimpanzees at 4°C and acidic pH (186). Norwalk virus VLPs did not agglutinate red blood cells (RBC) from
baboons, spider monkeys, Rhesus, chicken, guinea pigs, dogs, cats, mice, cows, pigs and rabbits which agrees with the lack of expression of HBGA on RBC from these species (see section 1.5.5). Agglutination occurred in all samples when Norwalk virus VLPs were incubated with human RBC from A, O and AB but only 4 of 14 from the B type. This assay was used as a model system for studying Norwalk virus attachment to cells in order to identify potential receptors. Convalescent-phase sera from Norwalk virus-infected individuals showed increased Norwalk virus VLP hemagglutination inhibition titers compared to prechallenge sera.

Wild-type and tissue culture SaV PEC/Cowden strain agglutinate pig erythrocytes to high titer (452). A hemagglutination-inhibition test was developed to quantitate antibody titers to PEC. The hemagglutination-inhibition and ELISA antibody titers to PEC were compared for hyperimmune antisera. Paired sera (pre-inoculation and convalescent) results from gnotobiotic (Gn) pigs inoculated with PEC/Cowden, and sera from conventional pigs, indicated that hemagglutination-inhibition and ELISA were complementary methods to quantitate antibodies against SaV PEC.

1.4 Epidemiology

The development of specific molecular diagnostic techniques revealed that human NoVs are the predominant agents causing viral gastroenteritis worldwide. They usually cause food-borne outbreaks, but can also be transmitted by personal contact, fomites and water (120). The use of these new molecular techniques also allowed the linkage and differentiation of outbreaks (9) and demonstrated that their prevalence is much higher than previously thought when many of the outbreaks where classified as unknown agents.
due to the lack of more sensitive methods (120). The U.S. Centers of Disease Control and Prevention (CDC) determined that NoVs were responsible for 96% of the nonbacterial gastroenteritis outbreaks in the U.S. in the period of 1996-97 (93). This epidemiological study using RT-PCR also revealed information on strain circulation over time and geographical distribution, settings, modes of transmission and age range. Similar conclusions were drawn from a study performed in Europe (258). The costs of treatment for gastroenteritis patients in Europe was estimated to be $200-300 million a year or an estimated 77 Euros per case (260, 437). In the study performed in the U.K. during 2002-2003, about 2,100 patients and 1,300 health care staff were affected in 227 gastroenteritis outbreaks causing the units to close and the staff to take leaves of absence. The NoVs were detected in 63% of the outbreaks. The information on costs of NoV gastroenteritis in developing countries is unavailable or unknown but could be quite different.

1.4.1 Molecular epidemiology

By the late nineties enough sequence information became available to observe the great diversity of the NoV genus. Sequence analysis is a robust tool to link and differentiate outbreaks (93, 238). It was by these means that a new strain was detected as predominant in the U.S. and U.K. and confirmed to have a global distribution with reports in seven other countries from 5 continents (110, 317). Globalization may have opened channels of trade and communication but also routes for transmission for pathogens. A well documented case involved raspberries grown in Bosnia and exported to Sweden and Canada, where sequences obtained from sick individuals were identical to
those recovered from the raspberries (242, 351). Another case where the source could be identified by molecular epidemiological investigation was one involving several outbreaks in the U.S. that were linked to oyster consumption. The oysters were traced to a single oyster bed harvested in a period of 2 days where a sailor had contaminated them by being sick on board (81). Since then, more emergent strains have been identified. In Japan a molecular epidemiological investigation found 96% identical NoVs in patients with gastroenteritis, oysters and the rivers flowing into the oyster farms (435). In 2002 the U.K. suffered a national epidemic caused by a strain from GII/4 (447). The new variant of GII/4 had a signature sequence in the RdRp gene that could be traced (256). Molecular characterization of NoVs showed that their mutation rate could help separate or connect the outbreaks (75). Identical sequences were linked in time or geographically in the U.K. These findings led to establishment of collaborative research and a surveillance network of viral gastroenteritis in Europe (256).

1.4.2 Prevalence

Age, genetic and environmental factors play a role in NoV infection. A study of Chinese military personnel (n=558) showed that seroprevalence to NoV VLPs from GI (Norwalk virus) and II (Mexico virus and VA387) varied depending on the blood type and the original residency (rural versus city) (63). The seroprevalence was 88.9, 54.1, or 90.0% for Norwalk, Mexico and VA387, respectively. Individuals of B blood type had significantly lower number of responses to Mexico virus whereas O blood type had the highest. The antibody titer to both Norwalk and Mexico antigen were also lower in B type individuals and this was the blood type of most of the Chinese students that did not
have antibodies to any of the 3 strains, suggesting people with this blood type are more resistant to NoV infection. The people coming from rural areas had higher rates of infection compared to people from the cities and people from the city had more individuals without antibodies to any strain. The blood type distribution did not differ between the rural and city areas.

A study in Canada showed that the seroprevalence of Norwalk virus was of 79% of the tested individuals (ages 9-60+) (341). Norwalk infections occurred in 33% of the individuals during the course of the study. This corroborated what has been described before, that previous seroconversion or a high serum anti-NoV titer is not always protective (see section 1.6.1). Another study demonstrated that seroprevalence to Norwalk virus increased with age, being 55% (ages 9-19), 79% (20-39), 87% (40-49), 84% (50-59), and 100% (60 and older) (341) and from 65-100% to Hawaii virus (60). By 50 years of age about 50% of individuals have Norwalk virus antibodies (232).

The higher seroprevalence to GII VLPs (Hawaii virus and Mexico virus) at a younger age when compared to Norwalk virus VLPs (GI) suggests that GII infections occur earlier in life in the U.K. However, seroprevalence studies in developing countries have demonstrated a different pattern of NoV antibody acquisition. A study of children in Bangladesh found that the prevalence of antibody to Norwalk virus increased from 7% to 80% in children of less than six months to children of 2-5 years of age (31). Similar results were observed in studies performed in Australian aboriginal children and children from Kuwait (74, 91).

In China the seroprevalence was 89% for Norwalk virus and 91% for Mexico virus (206). Similar seroprevalence between the two antigens was observed in individual
age groups. Infants had a seroprevalence at birth of 99% and 94% for Norwalk and Mexico virus respectively. The lowest seroprevalence, 30-40% for both strains, was at 7-11 months of age suggesting the fall in maternal antibodies. Then there was a sharp increase in seroprevalence in early childhood that reached almost 100% by the age of 10.

1.4.3 Incidence

The incidence of NoV gastroenteritis varies with age, being the major cause of viral gastroenteritis in adults but not in children where rotavirus is predominant (70, 101, 269, 341, 418). The NoV infection has been described in all age groups whereas SaVs are mainly agents involved in gastroenteritis in children, but they have recently been described as affecting adults too (318, 442). In a recent community-based study of human enteric calicivirus infections in Europe, researchers found that SaVs infected mainly children <5 years of age, whereas NoV infection was common in all age groups including children (364).

The incidence also seems to vary between developing and developed countries and it affects people with low and high income, although the sources of infection may vary. A study done in The Netherlands showed that the incidence of gastroenteritis due to viral pathogens was 21%, with NoV accounting for 11% of cases in all age groups. Bacterial pathogens accounted for 5% of cases, bacterial toxins for 9% and parasites for 6%. In community outbreaks, NoV was the leading cause. Similar values were observed in a study performed in Australia (269). From the 11.4% cases, 1.9% were caused by NoV GI and 9.6% were GII.
However in Indonesia the percentage was higher with NoVs detected in 21% of the cases of diarrhea, with 42% of these belonging to GI and 58% to GII (412).

In many cases, the pathogen responsible for an outbreak is not identified because of the lack of appropriate samples. The acute presentation of NoV presents a challenge because samples collected after the episode may become negative for virus particles. For this reason different strategies and collection devices have been tested. For example, a stool collection kit delivered to patients by mail demonstrated that in 96% of the outbreaks at least 1 person returned the kit with a sample to be tested (209). In total 76% of the people returned a specimen where 76% of the outbreaks were due to NoV and the rest (24%) to bacterial pathogens. The cost of the kit plus the shipping was of approximately $43 which included illustrated instructions, latex gloves, paper stool collection “hat”, labels, transport media, self-contained specimen spoon, sealable plastic bag, plastic transport container and prepaid mailing. Another less expensive and biosafe device tested for collection and transport and storage of fresh stool samples containing NoV was sodium dodecyl sulfate/EDTA-pretreated chromatography paper strips (465). The strips were tested after incubation at different temperatures (-80 to 37°C) and time (1-120 days) demonstrating that they were adequate for transporting NoV at ambient temperature for the time a sample could take to get to a laboratory to be tested by regular mail. Although the viral RNA could be tested, the infectivity was lost as demonstrated for FCV loaded into the SDS/EDTA-treated strips.
1.4.4 Host genetic factors for susceptibility or resistance

For decades it was not understood why some people were not susceptible to NoV disease even in absence of specific antibodies. It was also confusing that susceptible individuals usually had NoV-specific antibodies prior to infection (207). We now know that there are genetic characteristics in people that are responsible for the susceptibility and resistance to different NoV strains. The HBGA are at least part of the genes implicated in this process (see section 1.5.5). For example, a study using Norwalk virus showed that 20% of the studied population was non-secretor (Se−) which establishes that they don’t express HBGA such as the ABO(H) on their surface mucosal epithelia and therefore were resistant to Norwalk virus infection (249). These antigens may be receptor molecules for NoV (see section 1.5.5). These people, when challenged with Norwalk virus didn’t shed virus and didn’t have salivary or serum immune responses. For the Se+ population (80%); 45% became infected (all of them shedding virus), but only half of those infected manifested clinical symptoms. These people responded with late mucosal IgA and serum IgG antibodies. The other 35% of this group was protected, showing early IgA local immunity, no symptoms, no shedding and no serum IgG responses.

1.4.5 Transmission and sources

Although contaminated water and food are the main sources of NoV outbreaks (321, 322, 415), secondary person to person transmission is also well documented (122). There are multiple reports of probable airborne spread of NoVs by aerosolized particles during vomiting (47, 266, 267, 315, 377, 458). One study concluded that there was no evidence of flyborne transmission (56). Environmental contamination was demonstrated
after several cruise ships suffered consecutive outbreaks even after decontamination (457). The NoVs are quite resistant to disinfectants and transmission by fomites is also probable (see section 1.6.2) (83, 240). Some NoV outbreaks are caused by several strains suggesting foodborne sources (112). This has been demonstrated in shellfish, a common food involved with NoV disease and NoVs were detected in oysters in the U.S. market (354, 387, 414). Figure 1.5 shows the proportion of suspected modes of transmission for 90 outbreaks of non-bacterial gastroenteritis in the U.S. in 1996-7 (136). Also, there are cases in which transmission may have occurred during the pre-symptomatic stage (114) and the post symptomatic stage of the disease (454). Transmission routes might be suspected depending on the mutations found in different samples from the same outbreak. For example, no or few nucleotide substitutions are indicative of a point source whereas many mutations indicate person to person transmission (75). The NoVs have a low infectious dose of as few as 10-100 viral particles (1, 120, 128).

Although both outbreaks and sporadic cases occur, the first have been far more studied. Communal settings such as day care (5, 107, 277), schools (145, 177, 224, 267), summer camps, nursing homes (43, 102, 135, 199), hospitals (287, 346), cruise ships (229, 237, 240, 457), the military (12, 146, 276, 286) and events with catered foods (373, 425) are some of the most affected. Figure 1.5 depicts the percentage of settings involved in 90 outbreaks of non-bacterial gastroenteritis in the U.S. during 1996 and 1997 (136). Some of the identified risk factors are overcrowding, poor sanitation and raw shellfish consumption.
To date there is no confirmed case of animal to human transmission of NoVs. Although there were suspicious cases they were not well documented and characterized (182).

1.4.6 Geographical and temporal distribution

With slightly different incidence, NoV infections have been reported worldwide. This disease is present in developed countries from North America, Europe, Scandinavia and Australia as well as developing countries in Asia, South-east Asia, South America and Africa (51, 103, 178, 223, 274, 283, 348, 359, 408, 439). These countries have both hot and cold weather and rainy and dry areas. Different frequencies have been detected being higher in the coastal states of the U.S. (92) although this could be due to both environmental or behavioral factors such as seafood consumption. A more recent study of gastroenteritis caused by calicivirus for the 2000-2004 period performed by the Centers for Disease Control and Prevention (CDC) did not support this data (36). In this study, coastal states such as Florida, California, Oregon, South Carolina, Virginia and New York did not have outbreaks investigated by the CDC. In contrast, the states of Ohio, West Virginia, Maryland and Georgia as well as cruise ships had the highest incidence of outbreaks studied (from 14-39 outbreaks). The southwest states had 1-10 outbreaks investigated by the CDC whereas the north central states had none. Nevertheless, these findings are based on the report of outbreaks to the authorities and many cases are known to go unreported. Although transmission occurs year-round, seasonality has been demonstrated for NoVs with the majority of the cases indicating winter peaks (178, 256, 298), but with some exceptional peaks described for spring and
summer (259, 269, 341). In a study performed in Australia the NoV GI seasonality was significantly different from that of NoV GII (269). Two patterns of NoVs outbreaks have been described, one for health care institutions and one for community cases (257). For the health care facilities, the outbreaks were more prolonged in time, less likely to be of foodborne origin, caused higher death rates, involved fewer individuals and had a winter peak when compared to the community outbreaks. Molecular epidemiology investigations of these outbreaks resulted in data suggesting the virus was maintained in the health care centers by multiple introductions from the community, occasional transmission among wards and ongoing environmental contamination (75).

1.5 Pathogenesis in experimental and natural infections

1.5.1 Experimental or natural infection by caliciviruses

1.5.1.1 Humans Noroviruses and Sapoviruses

Human NoVs do not grow in cell culture and there is no animal model available hampering the study of their pathogenesis. Human NoV infections result in villus atrophy and malabsorptive diarrhea. This information was obtained from volunteer studies where mucosal biopsies were limited to the upper small intestine (4, 34, 80); therefore, information on other portions of the intestine are not available (86). Usually, histologic lesions correlate with diarrhea, but several reports described lesions in volunteers who did not show clinical symptoms (4, 80, 381). Even if vomiting is a common symptom, no histologic lesions have been described in the gastric fundus (460). On the other hand, there are numerous reports of asymptomatic individuals who become infected with NoVs
and shed virus through their feces (109, 124, 278). There are no pathogenesis studies of human SaVs, but a clinical volunteer study of SaV, showed that some adults manifest symptoms similar to NoV when inoculated orally or intranasally (58).

Recently, intestinal transplant pediatric patients diagnosed with NoV infection, presented secretory or osmotic diarrhea (219, 220, 297). Biopsies from these patient’s jejunum and ileum allografts showed infiltration of lymphocytes and plasma cells into the lamina propria, mild villus broadening and shortening, superficial epithelial cell disarray, nuclear enlargement, mucin depletion and increased glandular apoptosis. Apoptotic bodies were present in epithelial cells and macrophages containing apoptotic bodies were observed in the villus lamina propria. These lesions should be considered carefully taking into account that these individuals were taking immunosuppressive drugs that could have their own effect on the gut mucosa although they were compared to a control group of transplanted children without NoV infection. As a consequence of the immunosuppression, these patients had an extremely long period of diarrhea (17-326 days) that could make lesions much more severe and extended than in a non-transplanted individual. The NoV RNA detection remitted concomitantly after immunosuppression therapy reduction. Some of these lesions were common to allograft rejection but increased superficial apoptosis was characteristic of NoV enteritis.

1.5.1.2 Bovine NoV (Newbury 2-like) and unclassified NB-like strains

Two genetically distinct groups of BEC have been described, the NoVs comprising Jena and NA-2-like strains and the other unclassified group represented by the NB and the NA-1 strains (64, 252, 326, 388). Although both groups of BEC cause
diarrhea in calves they are not genetically related. Jena and NA-2 are classified within the 
*NoV* genus but in a separate genogroup than the human GI and GII (64, 252), whereas the 
NB strain is more closely related to the *SaV* and *Lagovirus* genera and probably 
represents a new genus in the *Caliciviridae* family (388). Another strain of BEC 
designated NA-1 was antigenically unrelated to the NA-2 showing heterogeneity in the 
capsid composition of BECs (39, 65). The NA-1 strain belongs to the same group as the 
unclassified NB strain (with 90% and 98% amino acid similarity for the RdRp and capsid 
protein, respectively) and genome organization into 2 ORFs (326).

When studying bovine NoV prevalence in dairy and veal farms in The 
Netherlands, no strong relationship between diarrhea and virus shedding could be 
demonstrated (438). From the veal calf farm pools, 31% were positive to the bovine NoV 
strains as well as 4% of the dairy cattle samples. The dairy cattle were infected at a young 
age (2.5 months). In Germany, a study using an antigen and antibody ELISA with Jena 
VLPs and hyperimmune antiserum to these VLPs detected 9% prevalence of Jena virus in 
dairy diarrhea samples (72). This low percentage is surprising when compared to the 
seroprevalence of 99% for this strain in one of the same regions. This suggests a high 
number of asymptomatic infections at a young age as described for human infants (see 
section 1.5.3). In the U.S., diarrheic samples from dairy calves from Michigan and 
Wisconsin contained bovine NoV (463). All farms tested in Michigan (n=8) and 2 of 14 
farms in Wisconsin tested positive to bovine NoV.
In Ohio, both bovine NoV and NB-like strains were detected (161). From 21 strains sequenced in the capsid region, 15 belonged to NoV GIII genotype 2 (GIII/2) and were genetically distinct from human NoV GI and GII. Six of 21 bovine strains had capsid gene sequences similar to that of the unclassified NB.

The bovine NoVs Jena strain, NA-2, and CV186-OH as well as NA-1 and NB cause clinical illness including anorexia, lethargy, and diarrhea in Gn (39, 466) or colostrum-deprived (CD) calves (72). The incubation period was 1-5 days (39). Prolonged shedding (60 days) has also been described.

Tillamook calicivirus was isolated originally in 1981 from dairy calves in Oregon; the finding of neutralizing antibodies in two widely distributed species of sea lions suggests the possibility of a marine origin for this agent (22). This calicivirus, which is a vesivirus, was isolated from 3 dairy calves in a herd with persistent calf respiratory disease problems. Experimental inoculation caused minimal lesions in 2 calves, but established a persistent infection with virus shedding for 45 days, time when the experiment was terminated. Experimentally exposed swine to this virus developed clinical vesicular lesions (394).

### 1.5.1.3 Porcine Sapoviruses and Noroviruses

Porcine enteric calicivirus Cowden strain was isolated from a piglet with diarrhea in 1980 (374). Experimental studies of Gn pigs inoculated orally or intravenously with SaV PEC/Cowden strain indicated that infection results in anorexia, occasional vomiting and diarrhea (99, 151) mimicking illness caused by human calicivirus. The SaV PEC/Cowden strain, causes cytolytic infections of the proximal small intestine in the
terminally differentiated villus enterocytes but not in the crypt enterocytes (153). The histopathological lesions in the pig’s tissues included broadening and blunting of the villi, crypt hyperplasia, cytoplasmatic vacuolization of enterocytes, and infiltration of polymorphonuclear cells into the lamina propria, whereas the mucosa remained intact (153). The increased number of crypt cells that secrete Cl⁻ is one of the mechanisms by which SaVs may cause diarrhea. It is unknown whether the recently identified QW270 and MM280 SaV more closely related to human strains that were recovered from feces of adult healthy pigs (449) can cause disease in young and naïve animals. Experimental and epidemiological studies to determine this are pending.

A porcine NoV isolated from healthy adult swine in the U.S., replicated in Gn pigs as detected by RT-PCR and seroconversion (451). One pig inoculated with QW144 strain shed virus from post-inoculation day (PID) 3 to 5 and experienced mild diarrhea. Intestinal contents collected at euthanasia on PID 5 contained NoV-like particles detected by IEM. Another pig inoculated with a closely related strain (QW126) did not have diarrhea but RT-PCR results were positive on PID 5. More studies are necessary to determine the pathogenesis of porcine NoV in pigs.

1.5.1.4 Murine Norovirus

A murine NoV (MNV-1) was recently identified (215). The use of RAG1-/- or RAG2-/- mice strains showed that these mice did not die after intracerebral, oral or intranasal inoculation of MNV-1. These mice were instead persistently infected showing viral RNA distribution in multiple organs (lung, liver, spleen, intestine, brain and blood) and shed virus in feces. Innate immunity plays an important role in protection against
MNV-1 as strains of mice that have an intact innate immune response are less susceptible. This hypothesis may explain the short symptomatology period (24 hrs) usually observed in human enteric calicivirus infections. Mice lacking interferon (IFN) α/β receptors, IFN γ receptors, PKR or iNOS were not more susceptible than wild type strains. In contrast, mice lacking both IFN α/β and IFN γ receptors had 10,000-fold increased risk of dying after MNV-1 inoculation by intracerebral or intanasal routes suggesting these receptors may be redundant. The fact that RAG/STAT -/- mice died whereas RAG-/- alone didn’t, together with the knowledge that STAT participates in the intracellular signaling following IFN type I and II stimulation suggested a critical role for IFN in resistance to MNV-1 infection. The quantification of MNV-1 RNA in different tissues of STAT-/- and wild type mice showed that the latter were infected after oral inoculation, with the viral RNA detected in intestine, liver and spleen, but this was cleared after 3 days. These mice also seroconverted but did not show any symptoms or tissue lesions. Infected STAT-/- mice showed a wider tissue distribution of the virus with substantial tissue pathology. The decrease in viral RNA after 7 days suggests that there is also a STAT-independent response. In another study MNV-1 RNA could be detected by RT-PCR in the spleen, mesenteric lymph nodes, and jejunum from some experimentally infected mice (not knockouts) 5 weeks post-inoculation (179).

The prevalence of this infection in mice from U.S. and Canada research colonies is of 22% (n=12,639) (179). This was determined by in vitro-propagated MNV-1 used as antigen to detect antibodies to MNV-1 in infected mice. This test had a specificity and sensitive of 100% in detecting anti-MNV-1 antibodies in sera from experimentally infected mice.
1.5.1.5 Feline calicivirus

The typical pathological lesions caused by FCV are in the tongue, soft palate and lungs corresponding to skin lesions and respiratory clinical signs. Virus multiplication is associated mainly with the tissues of the mouth and tonsils and the tonsil appeared to be the preferred organ of viral persistence in asymptomatic carrier animals (453). The FCV infection is seldom fatal; however, more virulent systemic strains of FCV have recently been described (183, 345).

The more virulent strains cause alopecia, cutaneous ulcers, subcutaneous edema, systemic vascular compromise and high mortality but other signs and lesions including bronchointerstitial pneumonia, and pancreatic, hepatic, and splenic necrosis were detected in some cases. Viral antigen was detected in endothelial and epithelial cells of the affected tissues by immunohistochemistry and viral particles were observed by transmission EM in the nucleus and cytoplasm of necrotic epithelial cells. Apoptosis of FCV-infected cells has been described in cell culture (see section 1.5.6), but its role in vivo is unknown.

1.5.1.6 Lapine RHDV and EBHSV

Rabbit hemorrhagic disease virus infection has been reported in Europe, Asia, South America and recently in the U.S. (2, 302). This disease is characterized by acute liver damage and disseminated intravascular coagulation (8). This virus infects hepatocytes, macrophages and endothelial cells as determined by immunohistochemistry of viral antigen in these cell types.
Apoptosis may be the process by which RHDV induced pathology in tissues (see section 1.5.6). Hepatocyte apoptosis produced parenchymal destruction causing a lethal, acute hepatitis. Apoptosis of intravascular monocytes and endothelial cells was observed together with fibrin thrombi in blood vessels and these might induce the pathogenesis of disseminated intravascular coagulation. There are also reports of non-pathogenic strains of RHDV (45).

The virus recovered from cases of European brown hare syndrome in the U.K. causes similar disease and shows some antigenic similarity, but transmission and protection studies failed to produce disease in rabbits and did not effectively protect against subsequent challenge with RHDV (52).

A new cultivable calicivirus was isolated from young European rabbits (Oryctolagus cuniculus) showing symptoms of diarrhea (273). Absence of neutralization by type-specific neutralizing antibodies for 40 caliciviruses and phylogenetic sequence comparisons of the ORF1 encoded polyprotein with those of other caliciviruses demonstrate that this new calicivirus is a new member of the Vesivirus genus which is closely related to the marine calicivirus subgroup.

1.5.1.7 In marine mammals

Calicivirus strains that infect marine mammals (pinnipeds and cetacean) belong to the Vesivirus genus. There are several serotypes isolated from vesicular lesions in sea lions and seals from Alaska to California (399). These viruses have also been recovered from aborted fetuses of seals suggesting that they can cause reproductive failure, although they were also isolated from healthy pups (396) which may indicate differential
susceptibility or existence of protective immunity. More recently, strains that were first
detected in terrestrial reptiles and amphibians were isolated from pinnipeds in California
(23). Another strain was isolated in cell culture infected with fecal matter from walrus
(113). Some of these strains may be zoonotic (See section 1.5.9).

1.5.1.8 Caliciviruses of other species (dogs, chicken, mink)

Canine calicivirus (CaCV) was first discovered in the feces of a dog with diarrhea
(378). It was easily propagated in cultures of canine cells and in a dolphin cell line.
Although seroprevalence is high and this strain was isolated from a dog with clinical
signs of diarrhea, its prevalence as a cause of disease is unclear. It was classified into the
Caliciviridae family by its morphology, physicochemical properties of virions, non
structural polyprotein and genome size, but it is distantly related to previously described
caliciviruses and it is closer to the Vesivirus genus (Figure 1.2.1). It did not react with
antisera to numerous caliciviruses except the stunting syndrome agent of chickens. The
seroprevalence of CaCV in the canine population of Japan is 57% (293).

The chicken calicivirus was obtained from gut homogenates from stunted broiler
chicks (59). It could be propagated in primary chick embryo fibroblasts. In vivo studies
showed that the RNA virus could be serially passaged in specific pathogen-free chicks
and virus particles were recovered from the gut and excreta of infected birds. The
morphological appearance and biophysical properties of the virus were similar to those of
FCV, which supports the view that it should be tentatively classified as a member of the
Caliciviridae family.
Mink enteric caliciviruses (MEC) were detected in feces from diarrheic mink by IEM (150). They showed classical calicivirus morphology with typical cup-shaped depressions on the viral surface. There is another type of mink calicivirus (MCV) with similar morphology, but it can be grown in cell culture in contrast to MEC, and the MCV does not cause enteric disease (150). The MEC and MCV were also detected by RT-PCR using a broadly reactive primer pair P289/290 targeting the highly conserved RdRp region of the enteric caliciviruses. Sequence analyses based on nucleotide and predicted amino acid sequences of the RT-PCR products indicated that MEC is most closely related genetically to SaVs of humans and animals. It shared 64-71% amino acid identities in the RdRp region with both human SaVs and SaV PEC/Cowden strain, 40-51% amino acid identities with vesiviruses and 29-33% with NoVs. Sequence analysis indicated that the MCV shared higher amino acid identities in the RdRp region with vesiviruses (58-81%) than with SaVs (43-51%) including the MEC, lagoviruses (35-37%) and NoVs (27-35%), suggesting that they are most closely related genetically to vesiviruses.

A calicivirus of unknown origin (designated isolate 2117) was isolated in Chinese hamster ovary (CHO) cells that manifested cytopathogenic effects in culture reaching moderate titers (323). Particles of about 40 nm in diameter with typical calicivirus morphology were observed by EM. The genomic characterization revealed an organization into 3 ORFs and the sequence identity was most similar to that of the CaCV, MCV and other caliciviruses within the genus *Vesivirus*. 

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Caliciviruses belonging to the *Vesivirus* genus have also been isolated from reptiles, amphibians and fish demonstrating the wide range of host specificity of this genus. These isolates resulted in infections when inoculated into mammals such as cattle, swine and monkeys (390, 397, 398).

### 1.5.2 Incubation time and clinical symptoms

In volunteer studies the incubation period for Norwalk virus disease ranged from 18 to 48 hrs, whereas clinical symptoms (mild fever, nausea, vomiting, abdominal cramping, and diarrhea) were generally self-limiting in nature and typically resolved within 24-48 hrs (124, 468). These symptoms are similar to those observed in disease outbreaks although only vomiting, only diarrhea or both were present in each outbreak and it might depend on the strain or most probably on the host individual characteristics. Acute severe dehydration can also occur (468). Virus shedding detected by IEM was maximal at onset of illness, and occurred only infrequently after 72 hrs but lasted for up to 13 days by ELISA (15). Only about 50% of adults exposed to Norwalk virus become ill (86). Illness caused by Hawaii virus and Snow Mountain virus in volunteers was clinically indistinguishable from that observed with Norwalk virus (263, 468). It was also noted that subclinical infections occur. The clinical symptoms for SaV-associated gastroenteritis were indistinguishable from those of Norwalk virus-infected patients (124, 133). In infected volunteers (380, 460), the incubation period for SaV was 12-72 hrs and illness lasted 1-11 days. The duration of detectable viral shedding paralleled the appearance of symptoms. Symptoms may also be affected by preexisting health conditions. For example, there is a report of British troops in Afghanistan where stress
and probably dehydration due to high environmental temperatures may have led to disseminated intravascular coagulation and a state of mental disorientation besides the typical vomiting, diarrhea and fever, symptoms that resembled the effects of some biological weapons. Another example are immunosuppressed individuals in whom clinical diarrhea can be prolonged for months or even years (see section 1.5.1.1). A report on a NoV outbreak in a facility for psoriasis treatment speculated that the clinical symptoms were more severe and with further health consequences because of the underlying disease of this group of people who suffered from a chronic disease that was treated with drugs such as steroids and methotrexate which affect the immune system (85). Noroviruses and SaVs may also be important pathogens in adults and children infected with HIV (62, 350, 367).

### 1.5.3 Symptomatic and asymptomatic infections

Both outbreak attack rates and volunteer studies indicate that there is a proportion of the population that is either resistant or protected from NoV infection (See section 1.4.4).

A study that tested a very small number of stools from asymptomatic people by RT-PCR, evaluated the role of asymptomatic people from the general population as a reservoir for NoVs (268). No NoV sequences were detected in the 400 samples tested. Another study that tested 532 control fecal specimens from adults by nested RT-PCR found 2 samples that were positive (331). Results were very different when testing asymptomatic people, both children and adults within an outbreak (109, 278). In a day care outbreak caused by a NoV, 11 of 14 children of less than a year of age had an
asymptomatic infection. In the following weeks 40% of children of this age group were infected (278). Gastroenteritis incidence due to NoV infections is low in infants (See section 1.4.3). This may be related to a high attack rate of predominantly asymptomatic infections in early life, resulting in a high prevalence of antibody to NoV by 4 years of age. In the case of adults, an investigation of a hospital outbreak of NoV gastroenteritis identified the causative strain as a NoV GII/3 (109). This strain was not detected in asymptomatic individuals. However fecal specimens tested positive for another strain of GII/4 NoV only by nested PCR, with 26% of asymptomatic staff and 33% asymptomatic patients shedding this virus.

Long term shedding after clinical resolution has also been reported in both children and adults (178, 270, 336) as well as long term shedding and clinical disease in immunocompromised patients (See section 1.5.1.1). A 2 year-old girl with no other health problems shed virus for 60 days after recovering from diarrhea with approximately 1x10^6 genomic equivalents per ml of stool suspension (178). Similarly an elderly woman continued excreting high levels (about 5 x 10^5 virions per gram of feces) of NoV GII, 3 days after gastroenteritis resolution (270). An outbreak investigation linked to sandwich preparation revealed the source of infection was a food handler who had recovered from diarrhea 4 days earlier (336). A specimen obtained from this individual 10 days after resolution of illness was still positive to NoV as well as a sample from another asymptomatic food handler.
1.5.4. Cell tropism, entry and replication

Carbohydrate antigens that determine the HBGA and other carbohydrate structures in the gut are NoV binding molecules (see section 1.5.5) (265, 419). Virus-cell interactions have been studied in vitro using differentiated Caco-2 cells which express H antigen. Norwalk VLPs can attach and internalize more efficiently to this cell line than to others (455). The binding is specific as shown by competition and blocking assays; however, only a small proportion (<7%) of the bound VLPs are internalized. The capsid region in Nowalk virus that is responsible for the attachment is a region located at positions 300 to 384 of the C-terminus of the capsid as there was total blocking when using MAb NV8812 which targets this epitope.

The replication cycle of NoVs is unknown because of the lack of cell culture systems and animal models for their study. The replication cycle of other members of the Caliciviridae family may have features in common but may also differ as SaV and Lagoviruses have a different genome organization. The replication of PEC (SaV) is dependent on the cAMP signaling pathway (48) (see section 1.5.8.1).

Sequence diversity at the 5’UTR of human NoVs could be responsible for differential regulatory signals (55). The La, PCBP-2 (poly(rC) binding protein 2) and PTB (polypyrimidine tract-binding protein, which has a role in RNA splicing) cellular proteins from HeLa and Caco-2 cell extracts bind to the first 110 nucleotides of the 5' end of Norwalk virus genomic RNA, a region previously predicted to form a double stem-loop structure (154). The protein-RNA interactions were determined using mobility shift and cross-linking assays. These proteins, which are important for poliovirus translation, are likely to play a role in Norwalk virus translation and/or replication. As described in
section 1.2.4, VPg of FCV serves as a “cap substitute” by physically interacting with eIF4E cap binding protein allowing FCV RNA translation (121). The ProPol fraction of FCV (or p76) interacts with VPg and may initiate viral RNA synthesis (212).

In vitro experiments using a non-structural protein encoded in the N-terminal region of ORF1 NoVs of both GI (Norwalk virus, GI/1) and GII (MD145, GII/4) has been associated with Golgi apparatus localization and disassembly of the Golgi complex into discrete aggregates (97). The protein responsible for the Golgi complex targeting was not identified but it may be p48, the homolog of p32 in FCV, which is an integral membrane protein that may form the core of the replication complex by interacting and anchoring other viral proteins in place (212). This resembles the function of the 2B protein of poliovirus (picornavirus), another virus with ssRNA of positive polarity. The 2B is known to associate to the Golgi and cause disassembly of the Golgi apparatus. Poliovirus also replicates in membranous compartments (97). In cells transfected with NoV replicons, two time-related patterns were observed: one of marked discrete aggregates and the other a more diffuse distribution of the protein throughout the cytoplasm. In FCV infected cells in vitro, replication was associated with cellular membranes (139). This was suggested by extensive rearrangements of membranes after infection when observed by EM and confirmed by isolation of membranous replication complexes. This fraction could actively synthesize viral RNA both genomic and positive and negative stranded subgenomic RNA. Nearly all virus-encoded structural and non-structural proteins were present in these replication complexes of FCV. These membranes may serve as a scaffold for packaging of the viral genome into the capsids that can otherwise self-assemble without RNA or VP2 (139). The product of ORF2 (VP1)
from FCV binds VPg, ProPol and VP2 as demonstrated by a yeast two-hybrid system (212). These interactions may result in encapsidation. In addition, the interaction of VP1 and VP2 may play a role in viral packaging. Subgenomic RNA is a source of additional message for capsid production. Short repeated sequences in the 5’ region of ORF2 of NoVs may play a role in regulation of subgenomic message production (55). The 3’-UTR of the genome is predicted to contain a stem-loop structure of 47 nucleotides. Proteins from HeLa cell extracts, such as La and PTB, form stable complexes with this region. The 3’ UTR with a poly(A) tail (24 nucleotides) permits the specific binding of the poly(A) binding protein (PABP) (155). Thus these host proteins may play a role in Norwalk virus replication or translation.

**1.5.5 Putative receptors: the role of ABO(H) and Lewis HBGA**

First established for *lagovirus* (371) and then for *NoVs* (185, 422), viruses in these 2 genera bind to HBGA. These antigens are carbohydrates and their synthesis is regulated by a group of genes coding for enzymes called glycosyl-transferases (*FUT1, FUT2, FUT3*, A and B) (358). These enzymes add a terminal monosaccharide to an oligosaccharide chain that is linked to a protein or lipid. These terminal sugars may be carried by all core chains known as Type 1,2,3 and 4 precursors (Figure 1.6 shows Type 1 and 2 synthesis pathway) (333). However there are ABO(H) antigen variants that arise from different immediate precursors (358). The *FUT1* and *FUT2* genes code for the H and the Secretor (Se) glycosyl-transferases which are responsible for the formation of the histo-blood group H. The activity of *FUT1* and *FUT2* also determines in which cells the H1 and H2 antigens will be expressed whereas H type 3 and 4 antigen expression is
independent of the \( FUT2 \) gene activity. In humans, individuals carrying an active \( FUT2 \) gene have the \( \text{Se}^+ \) phenotype, presenting ABO(H) antigens in various body secretions including saliva, milk, gastric juice and urine as well as on most epithelial cells of the respiratory, genitourinary, and digestive tracts (264). About 20% of individuals (of European origin) have homozygous \( FUT2 \) inactivating mutations and thus a non-secretor (\( \text{Se}^- \)) phenotype, or no ABO(H) antigens (especially H1) in secretions and on epithelial cells. The most common nonsense mutation (G428A) represents more than 95% of the inactivating mutations found in European and African individuals. This homozygous mutation segregated with complete resistance to NoV diarrhea in a study of 115 individuals (53 symptomatic and 62 asymptomatic) (429).

Some mutations such as A385T (missense) do not totally inactivate the gene but the expression is decreased resulting in a weaker secretor phenotype \( \text{Se}^w \) (332). Mutation frequencies vary depending on race. Many Asian people express \( \text{Se}^w \).

Swine have a homolog gene to the human Se, the guanosine diphosphate (GDP)-L-fucose:β-D-Galactoside α-2-L-Fucosyltransferase that was identified in the porcine submaxillary gland (431). No inactivating mutations of either \( FUT1(H) \) or \( FUT2 \) (Se) have been described in mammals other than humans (264).

The A and B glycosyltransferases are responsible for the A, B and AB blood types in humans and inactivating mutations in these give the O phenotype that means that the H antigen displays no further carbohydrate additions (358). Expression of ABO(H) HBGA on RBC has been a recent evolutionary event, being present only in humans and anthropoid apes (333). However these antigens are present on many other tissues including enterocytes of other mammals (333). In contrast to the 3 major alleles in
humans for the ABO(H) locus, pigs only have A and O(H) (471). The histo-group A transferase cDNA isolated from pigs shares a 77% nucleotide and a 66.7% amino acid identity with the human counterpart (289) and phylogenetic analysis of sequences from different species shows that they cluster together (42). Pigs also express A, H or I antigens in their gut epithelial brush border (16). The I antigen lacks the terminal fucose residue that characterizes the H antigens. Although the incidence of these pig phenotypes is unknown, from 10 brush border preparations, 4 tested A⁺, 4 tested H⁺ and 2 tested I⁺ (16).

The FUT3 gene codes for another glycosyltransferase known as Lewis (Le) that produces antigens that are independent from the ABO(H) antigens but are associated with Se phenotypes. These 2 genes, Le and Se, compete for the substrate chains and also interact by acting on the same product in a stepwise manner, so these carbohydrates can be secreted into body fluids in addition to their anchoring to proteins and lipids on the cell surfaces (Figure 1.6) (172). The FUT3 gene is highly active in the intestine and Lewis antigens are released as plasma glycolipids where they are acquired by RBCs that do not synthesize these antigens. The FUT3 gene, which encodes for the Lewis glycosyltransferase is not present in other mammals other than hominids (264); therefore, pigs should not have the Lewis antigens. Different forms of Lewis antigens associate with Se⁺ and Se⁻ people. The Se⁺ individuals can have Leᵇ and Leʸ and the Se⁻, Leᵃ and Leˣ (Figure 1.6) (172). The Lewis phenotype can vary in the first few years of life. By 2 years of age the Le phenotype is established for lifetime. There are different combinations of Lewis and the Se gene expression on RBC.
Distribution of HBGA expression in humans has been extensively described by Ravn et al. (358). For the small intestine, the superficial parts of the mucosal membranes express type 1 and type 3 chains mainly, whereas deep parts express type 2 chains. Expression of ABO(H) on the villi surface is confined almost exclusively to Se\(^+\); secretors express Le\(^b\), Le\(^y\); and non secretors Le\(^a\), Le\(^x\) on absorptive cells and goblet cells. The H type 1 and H type 2 can be expressed in the intestine of a Se\(^+\) individual but in different locations. In addition goblet cells express A/B and S-Tn (a type 3 chain antigen). In the crypts of Lieberkuhn, ABO(H) antigens are expressed without strict correlation with Se status whereas expression in the deep Brunner glands is completely independent of the \(FUT\) 2 gene and depends on the \(FUT\) 1 gene activity. Mucous cells express type 1, 2 and 3 ABO(H) and precursor structures in a secretor dependent way. Type 1 and 2 precursors are localized in mucinogen granules filling most of the cytoplasm; type 3 can be found in a supranuclear position suggesting localization to the Golgi apparatus. It was also demonstrated that A\(^+\) or B\(^+\) individuals may express A or B antigen in some acini whereas expressing H antigen in others, maybe due to different maturation stages. The RBC express mainly H type 2. Individuals with an inactivation mutation on the \(FUT1\) gene are referred to as the Bombay phenotype, although this is very rare (0.002% of the population worldwide) (221). However, the Bombay RBC have been very useful in the study of NoV VLP binding.

Norwalk virus VLPs bind to HBGA present on gastroduodenal epithelial cells of Se\(^+\) individuals (265). The \(FUT2\) gene (Se) was linked to differential susceptibility to Norwalk virus infection, where the Se\(^+\) phenotype determines susceptibility to Norwalk virus (248) and the Se\(^-\) phenotype are resistant to Norwalk virus infection (265, 448).
These results coincide with the finding that Se$^+$ volunteers are 40 times more likely to become Norwalk virus infected than Se$^-$ (248). A hemagglutination assay established that Norwalk virus VLPs show different patterns of agglutination of erythrocytes of different blood groups (186) (see section 1.3.8). These results coincided with the volunteer study findings (184) and suggest that B type individuals are partially resistant to Norwalk virus infection. It is now known that Norwalk virus binds H type 1, H type 2, H type 3, Lewis d (Le$^d$), Le$^b$ and Le$^y$ (169, 186) as determined by *in vitro* studies [hemagglutination (HA), hemagglutination inhibition (HI) and treatment with periodate and synthetic carbohydrates].

The VLP binding specificities were also corroborated by human challenge studies with Norwalk virus where Se$^+$ individuals with homozygous or heterozygous *FUT2* active genes were susceptible to infection and Se$^-$ individuals were not (249). This data was also supported by data of human retrospective studies (366).

*In vitro* studies using NoV VLPs from both GI and GII have demonstrated VLPs from different genoclusters have differential binding patterns for Lewis, Se and ABO(H) (180, 181, 422). This has also been confirmed *in vivo* (170). Even with the extensive data relating ABO(H) and Se phenotype with susceptibility and resistance to NoV, it is still unclear whether NoVs utilize HBGA as primary receptor or coreceptors (170). Some NoV GII strains may have two separate binding sites, the HBGA and heparan sulfate proteoglycan (419). Lordsdale virus, a strain belonging to GII/4 that has been the cause of the majority of recent outbreaks, has a broad specificity, binding to H type 1, H type 3, Le$^b$, A and B antigens (170).
The NoVs recognize HBGA in human milk samples (195). These antigens constitute 50-92% of the milk’s oligosaccharide content. Secretor and Lewis derived antigens present in human milk are responsible for blocking NoV binding to receptors; therefore, they may protect breast-fed infants from NoV infection. Four types of NoV VLPs from Norwalk virus (GI/1), VA387 (GII/4), VA207 (GII/9) and MOH (GII/5) were used to test the ability of human milk samples containing HBGA to block the binding of NoVs to saliva in an ELISA format. The proportion of Se⁺ and Se⁻ was 54 and 6, respectively. As expected from previous studies, all of the Se⁺ milk samples and none of the Se⁻ milk samples blocked VA387 and Norwalk virus binding to saliva from Se⁺. The VA207 binding was blocked by all Le⁺Se⁻ milk samples and variable blocking activities were exhibited by the Se⁺ milk samples. The MOH VLPs that bind to A and B antigens could not be blocked by any of the milk samples which is consistent with the fact that A and B antigens could not be detected in any of the milk samples. A recent report determined that two fractions of human breast milk from Se⁺ individuals could inhibit the binding of Norwalk virus VLPs to their carbohydrate ligands (372). These fractions were identified as the bile-salt-stimulated lipase and mucins. The mucins may act as decoy receptors as they contain HBGA whereas the bile-salt-stimulated lipase has a mucin-type C-terminal domain with 16 tandem repeats rich in potential O-glycosylation sites. The boiling of the milk did not affect the inhibitory activity suggesting the lipase activity may not be involved in the inhibition.

One report suggests that Norwalk virus VLPs bind to glycoproteins from swine gastric mucosa (432). An assay was developed to measure the inhibition of binding of Norwalk virus VLPs to HBGA in human saliva by porcine gastric mucin. The binding of
Norwalk virus VLPs to HBGA could be inhibited by porcine gastric mucin in a dose-dependent manner. Also, Norwalk virus VLPs could be captured effectively by porcine gastric mucin coated directly on plates and was detected by binding of polyclonal antibodies against Norwalk virus VLPs. Similarly, the binding of Norwalk virus VLPs to porcine gastric mucin was inhibited by HBGA in human saliva, and by Le\textsuperscript{b} and Le\textsuperscript{d} synthetic oligosaccharides, but was not inhibited by an H3 oligosaccharide or by purified bovine submaxillary gland mucin. Preincubation of Norwalk virus VLPs with porcine gastric mucin completely inhibited their binding to human Caco-2 cells. In this research the mucins from pigs and cattle were not typed, but they are likely to contain HBGA. The VLPs from NoV GII strain (SW918) from swine did not bind to human saliva (94). No data is available on pig saliva binding to human NoV VLPs. This study highlights the importance of further characterizing the Norwalk virus incidence and infections in animal hosts and the possibility that Norwalk virus is a zoonotic infection.

1.5.6 Virulence, apoptosis and persistence

The virulence factors of NoVs are not well understood but probably depend on both the virus and host characteristics (136). In recent years the strains that have emerged as most pathogenic have been from clusters with broad binding patterns to human HBGA as for example the GII/4 (447). This may be explained by the availability of more susceptible individuals in whom infection can occur. Host conditions such as health status as well as environmental conditions that affect the host, such as heat causing dehydration, may also affect the virulence of the disease (see section 1.5.3). At present there is no report of nonpathogenic NoV strains, but there are reports of nonpathogenic
RHDV and highly virulent FCV (44, 45, 183). A report on FCV suggests that variability in virulence among isolates may have a genetic basis as isolates responsible for certain types of syndromes such as hemorrhagic disease (similar to RHDV) clustered together when compared to isolates causing the typical respiratory and cutaneous signs of FCV infection (3). These more virulent strains may, in addition to viral invasion of epithelium and endothelium, elicit a different host cytokine response as demonstrated by a 3.8–fold elevation in IL10, TNFα and MIP-1α by real-time PCR from cDNA (100).

Another report comparing genetic and antigenic characteristics among isolates causing syndromes of chronic stomatitis, acute stomatitis, acute respiratory syndrome and limping syndrome failed to demonstrate a relationship between genetic relatedness and the syndrome presented (115).

An example of an atypical case of FCV infection occurred in 2 FCV-vaccinated cats after ovariectomy (71). No signs of respiratory disease but the presence of anorexia and depression and pustular lesions at the site of incision (abdomen) were evident. Immunohistochemistry results identified FCV as the pathogenic agent in both cases; one cat progressed to dyspnea so it was euthanized whereas the other cat recovered.

Little is known about SaV virulence factors except for the SaV PEC/Cowden in which the tissue culture-adapted Cowden strain has 4 amino acid substitutions in the capsid protein and 2 amino acid changes in the RdRp in comparison with the wild type strain (149). This tissue culture-adapted strain is attenuated when inoculated into Gn piglets (151). The specific amino acid/s that is/are responsible for the attenuated phenotype can only be identified by site directed mutagenesis followed by reassessment of their pathogenicity in Gn pigs.
Programmed cell death, also known as apoptosis, is a controlled mechanism involved in development, tissue homeostasis, and elimination of damaged or pathogen-infected cells (370). Apoptotic cells differ from necrotic cells in the following morphological features: DNA fragmentation, chromatin condensation, mitochondrial disruption and plasma membrane alterations. Induced apoptosis is an important way in which viruses cause disease, such as poliovirus causing apoptotic death of motor neurons (118); flaviviruses inducing apoptosis in neurons, endothelial cells, hepatocytes and mononuclear cells (7); and RHDV causing disseminated intravascular coagulation due to intravascular death of monocytes (8). Although apoptosis is a physiological process, alterations such as increased and decreased number of apoptotic cells or the occurrence of apoptosis in a cell population (for example neurons) or a location (for example intravascular) may result in tissue damage and disease (370). Apoptosis regulation is targeted by many viruses including cytomegalovirus, poxviruses, African swine fever, Sindbis, human immunodeficiency virus (HIV), poliovirus, flaviviruses, herpesviruses and adenoviruses (7). Apoptosis occurs through 2 pathways: the extrinsic and the intrinsic pathways (129). The extrinsic pathway is initiated by the tumor necrosis factor (TNF) family of death receptors, where activation-induced cell death ligands bind to their receptors. The receptor’s death domains in turn bind to the pro-caspases 8 and 10, a family of cysteine proteases with aspartate-specificity, forming a death-inducing signaling complex and activation of caspase 3. In addition, infected cells suffer the attack of cytotoxic T lymphocytes (CTLs) through the recognition of peptides expressed in the MHC-I by the TcR in the CTLs. The granzymes secreted by CTLs also activate the caspase cascade in the infected cells. The intrinsic pathway is activated via internal
sensors such as p53 and by the Bcl-2 related proteins which cause pores in the membrane of mitochondria, propagating the signal. The release of cytochrome c and the second mitochondria-derived activator of caspases (smac) into the cytoplasm promotes the formation of the apoptosome by interacting with the apoptosis-protease activator 1 (Apaf-1) and the autocatalytic activation of caspase 9 that initiates the caspase cascade with the activation of caspase 3.

Increased superficial apoptosis has been described as a typical pathological finding in NoV-infected intestinal-transplanted pediatric patients whereas apoptosis in the crypts was common to both NoV infection and tissue rejection (see section 1.5.1.1). Apoptosis of hepatocytes, macrophages and endothelial cells was observed in naturally and experimentally RHDV-infected rabbits with severe pathology usually resulting in death. Apoptosis may be a determinant in the development of the pathogenesis of RHDV disease (8). Apoptosis has also been described for FCV infection of cell cultures (6, 363, 407) and it requires active viral replication for induction of apoptosis (407). Replication occurs in the cytoplasm where membrane rearrangements produce numerous intracellular vesicles (97). This could be a consequence of caspase 3 activation involved in cellular disassembly. The mechanism by which FCV induces apoptosis has been partially defined as a result of activation of the intrinsic pathway with loss of mitochondrial membrane potential at 4 hrs post infection, followed by the translocation of phosphatidylserine to the cell outer membrane and the release of cytochrome c from the mitochondria at 6-8 hrs post-infection (306). The extrinsic pathway may not be involved since a caspase 8 inhibitor did not inhibit or delay apoptosis in the FCV-infected cells. However, it was also demonstrated by others that after FCV infection caspases 8 and 9 (407), 2, 3 and 8
are activated. Coincidently with the FCV infection and caspase activation, cleavage of the capsid protein of 62 kDa (VP1) into a smaller 40 kDa occurs (6). This phenomenon of cleaved particles has also been observed in RHDV and as soluble antigens of Norwalk virus detected in stools of diseased individuals (168). Apoptosis may be a defense mechanism of the host by sacrificing an infected cell to limit viral dissemination. It is also possible that FCV induces apoptosis to replicate, disseminate the viral progeny or to avoid the host’s immune response (426). Caspase-mediated capsid cleavage may interfere with virus assembly and reduce virus yields; alternatively, these smaller proteins may act as free antigen generating non-neutralizing antibodies favoring viral infection (168). The FCV infection of a feline kidney cell line (CRFK) also induced inhibition of host cell protein synthesis by cleavage of eIF4G initiator factor in a similar but distinctive manner as picornaviruses and this was independent from caspase-mediated apoptosis (461).

Persistence and chronic infections have been observed in human NoV infections after disease recovery and in immunocompromised individuals, respectively (see sections 1.5.3 and 1.5.1.1). Both the immunosuppression as well as the immune pressure selecting for virus mutants may be responsible for such events (see section 1.5.7).

Feline calicivirus can cause persistent infections (356). The evolution of the hypervariable region of the FCV capsid gene was studied both in vitro and in vivo. This region of the capsid protein contains neutralization epitopes and may be a target for immune evasion during virus persistence in the host. Sequence analysis showed that FCV exists as a quasispecies which evolves both in cell culture and in persistently infected cats. The quasispecies have more than 92% sequence similarity in opposition to mixed infections where nucleotide differences among FCV strains range from 57-79% (355).
After 90 passages in cell culture and during replication in persistently infected cats for 39 days, changes such as deletions and a gain of both synonymous and non-synonymous nucleotide substitutions were detected. Overall, these changes led to a reduction in population heterogeneity over time both in cell culture and in infected cats. Evolution rates for the consensus sequence ranged from 0.10 in an immunosuppressed cat (infected with feline immunodeficiency virus) to 1.07 substitutions per nucleotide per year, in normal cats. Marked changes in virus neutralization profiles were seen in isolates obtained sequentially from a persistently infected cat. This was not the case with cell culture passaged virus, suggesting that the individual amino acid changes found only in virus from persistently infected cats may significantly alter the antigenic profile of FCV, and may be the result of immune selection (208, 342). Chronic infection has been described for RHDV infections in which smaller particles designated core-particles might result from defective replication (125).

1.5.7 Viral evolution

The NoV evolution is driven by mutations that involve cumulative point substitutions, insertions or deletions as well as recombination (369). In general RNA viruses have a mutation rate of approximately $10^{-3}$ (whereas for DNA viruses it is $10^{-9}$) because the RdRp in RNA viruses have no proofreading activity (410). In the case of RNA viruses, every population is composed of varied genome alternatives (quasispecies) whereby one clone is predominant under the existing conditions because it is better fit (393). When conditions change (for example in an alternative host) other clones may be more fit to the new conditions and a different clone will dominate. Evolution is rapid
because the new clone already existed but was not expanding because of disadvantageous conditions. Quasispecies have been described for the FCV (356), RHDV (123) and other RNA viruses (410); therefore, it is likely to be the case for NoV populations. However because there is no cell culture system to grow human NoV, it has not been possible to confirm these characteristics for this group of viruses.

The selection pressures that viruses encounter are both the stability in the environment (temperature, humidity, pH, salt concentration, etc.) and within the host where there are multiple barriers that viruses need to overcome with the immune system being of major importance (411). Evolution in higher organisms occurs at a lower rate because, even if the mutation rate is the same as that of DNA viruses, the size and complexity and the presence of repair systems limit the flexibility of higher organisms (410). Viruses are extremely flexible (progeny numbers, replication time) and back mutations occur frequently. Passage in a new host imposes tremendous pressure and only the clones able to adapt will remain even if this means losing virulence in the former host. The virus evolves into a new niche, but its genome is unstable until a dominant phenotype establishes into the new host environment. This is what is necessary to occur in order to adapt a NoV to a new host.

Cumulative point mutations are frequently seen in NoV outbreaks (75) and are very useful for epidemiological research (see section 1.4.1). There are well described cases in which viral mutants were observed within an individual with prolonged shedding. An exceptional case of a chronic infection lasting 2 years involving an immunocompromised adult, provided valuable information on the actual (not predicted) mutation rate of NoV (313). Sequence analysis of the capsid gene over time revealed 32
amino acid changes during one year. Most of the cumulative amino acid changes (8 of 11) were located in the P2 domain exposed to immune pressure. These accumulated amino acid changes in the surface P2 domain resulted in predicted structural changes and thus a possible emergence of a new phenotype. Mutations in the capsid region may not only evade the immune system but also change the receptor specificity as demonstrated by *in vitro* studies using VLPs and HBGA (421). The high mutation rate was also demonstrated by four mutations and three microheterogeneities in 3,255 nucleotides during 17 days of NoV shedding by an immunocompromised patient (75). In the general human population, during a 3 year period (1996-99) the consensus sequence of Lordsdale-like strains changed 3% in the RdRp region (231).

The increased number of outbreaks during 2002 and 2003 may be explained by alterations to the capsid structure compared to previous NoVs. The NoV epidemic strain differed from its closest previously described relative by 11.4 to 13.6% in the outer P2 domain of the capsid, which also had a single-amino acid insertion (75).

Several reports of naturally occurring NoV recombinant strains (166, 193) indicate that this may be a common phenomenon although the mechanism cannot be studied in the absence of a cell culture system. During recombination the RdRp might jump from one template to another by a copy choice mechanism with the ability of the RdRp to detach and attach to a new template. The process was well established in the murine hepatitis coronavirus and poliovirus (411) but has not been proven for NoV. The NoVs have a subgenomic RNA (see section 1.2.3) that may facilitate recombination by this mechanism. Recombination within the *NoV* genus can affect their classification when done using the RdRp region compared to the one that would be obtained when using the
capsid sequence as the recombination usually occurs between ORF1 and 2 (193, 218, 253, 443). A strain designated Arg320 was sequenced from a 3.3 kb cDNA from the RdRp region to the 3′ end of the genome. The capsid region shared 95% amino acid identity with Mexico virus, but 68% identity with Lordsdale virus, whereas the RdRp region shared 95% identity with Lordsdale virus, but 87% identity with Mexico virus. Pair-wise sequence comparisons identified a potential recombination site at the RdRp/capsid junction. Recombination has only been described for strains within a NoV genotype. Recombination may be associated with mixed infections. Although this has not been proven it is reasonable to think that when multiple strains are concentrated by shellfish, present in fresh produce irrigated with contaminated sewage, detected in the same outbreak, cocirculating in the community or even coexisting in an enclosed environment such as a ship or a hospital by symptomatic and asymptomatic shedding of patients and personnel (108-111, 126, 211) there is a high probability of a dual infection and thus of recombination (376).

Recombination may also occur within the NoV capsid gene. This was evaluated in 94 complete ORF2 sequences where recombination was detected in about 8% of NoV strains (369). From these recombinants, one was GI/1 strain, one was a GII/1, one was a GII/3, three were GII/4 and one was a GII/5. Recombination sites were located at the interface of P1 and P2 domains of the capsid protein and/or within the P2 domain. The recombination region displayed common features such as length, sequence composition (upstream and downstream GC- and AU-rich sequences, respectively), and predicted RNA secondary structure that are characteristic of homologous recombination activators in the capsid domains that may be exposed to immunological pressure.
Recombination has also been observed in NoVs from other species. A potential porcine NoV recombinant genotype has been described (451). This genotype, comprised of 2 strains (QW218, QW170), clusters closer to the previously described Sw43 strain from Japan when based on the RdRp partial sequence than when based on the complete capsid nucleotide sequence. It asserts into a different cluster when comparing the ORF2. The result of the Recombination Identification Program (RIP) suggests that the RdRp/capsid junction is the recombination site. This area contains a highly conserved 18 nucleotide motif that probably plays a role in genomic/subgenomic transcription (218). This site was previously identified as a recombination site for the Arg320 strain (193).

The existence of chimeric genomes in bovine GIII NoVs suggests that genomic recombination is part of the natural evolution of NoVs and is relevant to the diagnosis and immunological control of NoV diarrhea outbreaks (328). The bovine NoVs, NA-2 and Jena, represent two clusters in NoV GIII but a chimeric virus at the RdRp/capsid region between these 2 strains has been identified. This strain was designated Thirsk and has a Jena-like RdRp gene but a NA-2-like capsid and ORF3 genes based on comparison of their nucleotide sequences and phylogenetic analyses. Furthermore, the bovine NoV strain CV521 showed high nucleotide and amino acid identities (84 and 94%, respectively) with the capsid gene of NA-2, whereas the nucleotide and amino acid sequences of the RdRp gene were more closely related to those of Jena (77 and 87% identities, respectively) than to those of NA-2 (69 and 69% identities, respectively), suggesting that CV521 strain is a bovine NoV GIII, cluster 1-2 recombinant (161).
Gene conversion analysis by the RIP and SimPlot programs also predicted CV521 strain to be a recombinant. This information provides additional support that genomic recombination is part of the natural evolution of NoVs.

Sequence comparison of SaV genomes from strains isolated in a 10 year period suggests that this genus is more stable genetically based on its high level of sequence identity (192). Porcine SaVs also recombine (449). Two recently identified strains mapped to different location in the neighbor-joining phylogenetic tree constructed using the complete capsid sequence versus the partial RdRp sequences. The QW270 and the MM280 clustered together with the PEC/Cowden strain (90% sequence identity similarity) based on the capsid sequence, but they had only 76-83% similarity when comparing the partial RdRp sequence and they differed from each other in this region. The recombination site was also at the RdRp/capsid junction although these are both located within ORF1 in the SaV genus in contrast to the ORF1-2 localization in the NoV genome. The POY analysis (Phylogeny Reconstruction via Optimization of DNA and other Data) was used to confirm that a recombination event rather than accumulation of point mutations was responsible for the identified phenotypes. Recombination has been described for the human SaV within a genogroup (217) and more recently between genogroups II and IV (164) at the RdRp/capsid junction. This finding raises the question of possible recombination between human strains and porcine strains belonging to SaV GIII.

Another way of evolving based on defective interfering particles has been described for other RNA viruses (73). Although it is unknown if NoVs have interfering particles, they have been suggested for RHDV when causing chronic liver disease (125)
and in FCV both in cell culture and in persistently infected cats (356). The defective interfering particles have been described for vesicular stomatitis virus, rabies virus, Sinembis virus, West Nile virus and lymphocytic choriomeningitis virus (73). The defective interfering particles carry mutant genomes. These defective genomes need the help of normal viral genomes to replicate. These genomes can act as highly flexible evolution modules that can tolerate large deletions or sequence rearrangements which may in the future be inserted back into a complete genome by recombination resulting in a major drift in the phenotype (73). In summary, viral evolution causes changes that permit viruses that were restricted to a niche to expand or generally change this niche (tissue, host), sometimes causing a new or more virulent disease (410, 411).

1.5.8 Pathogenesis studies

1.5.8.1 In vitro: Cell culture and infectious clones

To date no human NoV strains can be grown in cell culture. Many attempts have been made, but all have failed with some being published and many more that were not (84). Organ culture as well as many cell lines of human, monkey, feline, rat, mouse, canine and bovine origin were tested under various conditions, with different additives (proteases, hormones and intestinal contents) and using several NoV strains of different clusters within both GI and GII (84). Some cell lines (13 cell lines from different origins) showed attachment of Norwalk VLPs, such as differentiated Caco-2 cells (a colon cancer derived cell line) (see section 1.5.5).

The only SaVs that can be grown in cell culture is the PEC/Cowden strain that belongs to a separate genogroup (GIII) from the human GI and GII. This strain was first
adapted to growth in primary porcine kidney cell cultures with the addition of porcine intestinal contents (98) and then in two continuous porcine kidney cell lines (340) (LLCPK and PK-15 derived from epithelial-like cells in the proximal renal tubule). The PEC/Cowden cell culture system permitted identification of a cAMP signaling pathway induced by the addition of intestinal contents for the initial replication of PEC, and should be useful in the study of antiviral drugs (48). It was also established that bile acids in the intestinal contents might be responsible for the virus infectivity by increasing cAMP and subsequently decreasing interferon-mediated STAT-1 in LLC-PK cells (50). Therefore, the intestinal contents containing bile acids required for PEC/Cowden replication might induce a protein kinase A (PKA) signaling pathway and downregulation of innate immunity.

The recently discovered murine NoV (MNV-1) can be grown in primary cultures of macrophages and dendritic cells (464), and RAW cells (a macrophage derived cell line). In vivo, this strain infects macrophage-like cells. Although the cell/tissue tropism of MNV-1 (that has been temporarily classified as NoV GV) differs from the human NoV strains, this system is useful to study the replication of the virus which is still poorly understood.

Another research approach to study these viruses replication consisted of constructing an infectious clone of Norwalk virus that could replicate in human embryonic kidney (HEK) 293T cells (14). Intracellular expression of Norwalk virus RNA devoid of extraneous nucleotide sequences was obtained from an expression vector by using a replication-deficient vaccinia virus encoding the bacteriophage T7 RNA polymerase. The viral genomic RNA replicated and subgenomic viral RNA was
transcribed by Norwalk virus nonstructural proteins. However the viral capsid protein could only be detected after expression from a helper replicon that was subsequently translated into Norwalk virus VP1. Viral genomic RNA was then packaged into virus particles that, when purified, were similar to native particles from stools. This approach was previously described for the FCV infectious clone but the replicon was also defective and could only be packed into virus particles when the structural proteins were added \textit{in trans} (430). Although FCV (\textit{Vesivirus}), RHDV (\textit{Lagovirus}) and PEC (\textit{SaV}) can be grown in mammalian cells, infectious clones of these viruses have helped to elucidate their replication strategies, including polyprotein processing and cleavage sites (49, 291, 430).

For the PEC infectious clone, a full-length cDNA copy of the Cowden PEC genome was cloned into a plasmid vector directly downstream from the T7 RNA polymerase promoter and transcripts were then capped in order to become infectious. The recovery of PEC particles from LLC-PK cells after transfection of RNA transcripts was dependent on the presence of bile acids. The recovered virus was infectious to orally inoculated piglets resulting in mild disease similar to that of the parental tissue culture PEC/Cowden.

Recently, the complete genome of a human NoV GII strain was cloned into a vaccinia virus expression system under the control of a T7 RNA polymerase promoter and expressed in human embryonic kidney 293T/17 cells (216). Positive and negative polarity RNA segments of 7.6-kb and a 2.6-kb positive-strand subgenomic-like RNA were detected in infected cells. However, no viral particles were recovered from supernatants and structural proteins could not be detected by Western blot.
In this system, when VP1 and VP2 genes were expressed from a co-transfected construct, virus particles were observed but their size was variable (20-80 nm); thus, their antigenicity and structural morphology need to be confirmed.

**1.5.8.2 In vivo: Volunteer studies and experimental animal models**

Most recent human volunteer studies assessed immunity to NoV (see section 1.6.1). Information on pathogenesis of NoV in humans comes from outbreak investigations, NoV-infected immunosuppressed transplant recipients and earlier human trials (see sections 1.1.2, 1.5.1.1, 1.5.2).

Trials involving animals inoculated with human NoV strains have resumed in recent years. Toronto virus (NoV GII/3) was inoculated into five pigtail macaques and all shed virus in their feces and 3 of 5 showed clinical signs, although the lack of Toronto virus specific antibody responses was difficult to explain (413). In a more recent study, common marmosets, tamarins, cynomolgus and Rhesus macaque monkeys were orally inoculated with Norwalk virus (365). A low level of replication may have occurred in marmosets and tamarins in which Norwalk virus was detected in feces for 3-4 days after inoculation, but neither clinical signs nor antibody responses could be detected. Cynomolgus monkeys were not susceptible to infection, in contrast to the Rhesus macaques, with all 3 inoculated animals shedding virus in feces (2 for 1 and 2 days and another one for 19 days). This Rhesus macaque seroconverted to Norwalk virus whereas the other 2 Rhesus macaques did not. No clinical signs were evident in any of the animals including the Rhesus macaque with the long period of shedding. All animals were Se+ and the Rhesus macaque that shed for 19 days post inoculation was A+, whereas the other
2 were B$^+$ and H$^+$ respectively. Marmosets were A$^+$ or AB$^+$, tamarins were AB$^+$ and cynomolgus monkeys were A$^+$ or B$^+$. In this same study the investigators evaluated the seroprevalence of Norwalk virus and virus shedding in chimpanzee colonies and concluded that these apes had not been previously exposed to Norwalk virus. This finding represents a partial discrepancy to another study where a 92% prevalence rate of NoV antibody was detected in a primate research center (190). The different result could be explained by the different primate colonies tested with differing exposure histories, the different species of monkeys (4 old and 1 new world) and the different virus strains (Norwalk on G1/1 and GII/4 and GII/7), used for the antibody assays. None of the capuchin monkeys’ (new world) sera tested positive but, 92% of the chimpanzees’ sera (n=12) had Norwalk virus antibodies. Fifty-three percent of the mangabey, pigtail and Rhesus macaques (n=83) had antibodies to G1/1 and 58% had for GII/4 and 7. In another study performed using newborn pigtail macaques inoculated with Toronto virus, clinical illness was manifested by 2 of 3 monkeys in group 1 (inoculated with the original human stool filtrate) and 1 of 2 monkeys in group 2 (inoculated with the filtered feces from group 1). All 5 monkeys shed virus in their feces. In the first group, one monkey that did not develop clinical signs shed virus in its feces from day 1-14 by RT-PCR and hybridization and days 7 and 14 by antigen ELISA. The second monkey had only one day of diarrhea on day 2 post inoculation but was negative by RT-PCR (except for day 21) and antigen ELISA but positive by hybridization of RT-PCR products on days 1, 3, 7 and 21 possibly indicating a low amount of virus shedding. The third monkey in this group had diarrhea on days 2 and 3, and was positive by RT-PCR and hybridization from days 1 to 21 with the exception of day 7 (by RT-PCR) and was positive by antigen ELISA on
days 7, 14, 21, 28. In the second group, again the monkey with diarrhea on day 3 shed virus from days 1-21 by RT-PCR and hybridization but shedding was not detectable by antigen ELISA. The monkey with no clinical signs shed virus for the same amount of time (detected by RT-PCR and hybridization), but virus antigen was detected on days 7 and 14 by antigen ELISA. This long asymptomatic shedding has been described in humans where 28% of infected individuals shed virus for up to 3 weeks after onset of disease (364). Two parent monkeys kept in the same cage as their inoculated offspring became ill and infection was confirmed by detection of Toronto virus RNA in their stools demonstrating secondary infection. Not all Rhesus macaques seroconverted as measured by antibody ELISA, although these results should be interpreted with caution as the antigen used in the assay was Norwalk virus VLPs belonging to GI/1 NoV whereas Toronto virus belongs to GII/3 where little crossreactivity exists. It is unknown why the investigators used Norwalk virus VLPs and not other VLPs from GII which are also available (Table 2). When they used Toronto and Norwalk virus VLPs, these may have failed to detect seroconversion because of the presence of maternal antibodies or because antibody titers could have been below the detection limit of the test. Similar results have been reported in Rhesus macaques where infection of Norwalk virus occurred in both the presence and absence of seroconversion (57). Virus particles were seen by EM in feces of 2 Rhesus macaques, but none were detected in other individuals. In the experiment by Rockx et al. (365) only 1 of 3 Rhesus macaques seroconverted coinciding with a long period of virus shedding. Interestingly, IgM and IgG antibody responses were concomitant with virus clearance but no IgA could be detected in either plasma or saliva in opposition to what has been described for symptomatic and asymptomatic humans and
chimpanzees (124, 469). Based on Norwalk virus and Grimsby virus VLP binding assays to RBCs and the ABO(H) and Se typing of these species, the researchers concluded that the VLP binding capacity of RBC did not correlate with susceptibility to NoV infection in non-human primates (365). Norwalk virus VLPs did not hemagglutinate Rhesus macaques RBCs whereas Grimsby VLPs did. However, infection of Rhesus macaques occurred with Norwalk virus and unfortunately Grimsby virus was not inoculated to Rhesus macaques so no information is available for that strain. The duration of shedding for 3-4 days in marmosets and tamarins is similar to the findings for humans and chimpanzees. These findings together with the fact that no virus shedding was detected in cynomolgus monkeys and shedding was 1-2 days in the other 2 Rhesus macaques suggest limited replication. Furthermore, UV-inactivated virus was only detected till day 2 by RT-PCR although with a 10-fold reduction. Treatment with proteinase K and ribonuclease before RT-PCR were efficacious in avoiding positive detection of inactivated viruses such as FCV, poliovirus, hepatitis A (319).

1.5.9 Interspecies transmission and zoonosis

Molecular characterization of a few animal NoV and SaV strains initially indicated that porcine SaVs and bovine NoV belonged to different genogroups of the human SaV and NoV genera and as members of different genogroups, they posed little threat to human health (149, 329). However, the existence of antibodies to bovine NoV GIII in the human population suggests that humans may become infected with bovine NoV strains although the infections might be asymptomatic. A serological study performed in Europe using a bovine NoV GIII-specific antibody ELISA showed the
presence of serum antibodies to the bovine NoV strains in humans (459). Twenty-two percent of the sera tested (n=840) had IgG antibodies to bovine NoV GIII VLPs. Although there was some cross-reactivity between bovine NoV GIII and GI NoV, but not with GII NoV, 26 sera showed high reactivity to bovine NoV and low reactivity to GI NoV suggesting that the response to the bovine NoV antigen was specific. Interestingly veterinarians had a significantly higher GIII antibody seroprevalence (28%) when compared to the rest of the population (20%) probably due to a higher risk of exposure.

In addition, the detection of antibodies to human NoVs in swine raises additional questions on the role of pigs as reservoirs for human strains (94). Of 266 pigs tested in the U.S., 63% had antibodies to Norwalk virus (GI/1) and 52% had antibodies to Hawaii (GII/1). Moreover, the identification in pigs of NoV and SaV strains that are genetically closer to human strains and are antigenically related, in the case of the NoVs, exposes a potential zoonotic risk (94, 449, 451). Swine may act as reservoirs of new strains for humans or may be accidental hosts of human strains, but mixed infections could result in human/porcine recombinants.

There are also reports of interspecies transmission of vesiviruses (401). An isolate initially infecting an Atlantic bottlenose dolphin, spread to a California sea lion, which when moved to another facility infected a second dolphin that developed vesicular skin lesions. Cetaceans and pinnipeds belong to separate orders, demonstrating that vesiviruses have a broad host spectrum. The vesiviruses infect a phylogenetically diverse array of hosts including fish, reptiles and mammals. A sea lion vesivirus isolate (391) that replicated in primate and human cell lines, induced type-specific neutralizing antibodies in exposed humans. These antibodies reacted with San Miguel sea lion virus (SMSV)
virus particles by IEM. Group antibody against a pooled antigen of SMSV-5 and two other serotypes were also observed in 18% of 300 blood donors from a population in the northwestern U.S. (391). In addition, a laboratory worker became infected with an isolate recovered from a sea lion. The worker developed systemic illness, including vesicular lesions on all four extremities and viremia (391). A recent study of the seroprevalence of vesiviruses in the human population (n=765) revealed that 12% of the blood cleared for donation, 21% of the blood not accepted for donation because of elevated blood liver alanine aminotransferase (ALT) concentration, 29% of blood from patients with signs of hepatitis suspected to be of infectious origin but of unknown agent and 47% of blood from people with clinical hepatitis of unknown origin but related to blood transfusions or dialysis were positive by antibody capture ELISA using 3 types of SMSV (one detected in a human case, one known to cause disease in livestock and one recovered from edible shellfish) and FCV F9 live vaccine strain antigens representing different possible exposure settings to humans (392). Vesivirus SMSV-5 specific-antisera has broad cross-reactivity to other vesivirus serotypes but not with Norwalk virus (391). In addition, vesivirus viremia was detected by dot blot, RT-PCR and sequencing in 11 of 112 sera tested. Primers used in two different laboratories targeted the RdRp and the capsid respectively. Five of the RT-PCR-positive samples were also antigen ELISA-positive and six were not. The sequence data indicated that five strains that were closely related (1-6% divergent) were most similar to PAN-1 strain (88-96% identity) and SMSV-6 (88-94% identity) and only 84-86% similar to SMSV-5. One strain, designated N104 had 68% identity to SMSV-13 and 44% to F9 in the RdRp region and 97% identity to SMSV-5 in the capsid region. Thus, vesivirus may infect humans not only accidentally in the
laboratory setting but also infect the general population more frequently than previously thought and may be responsible for hepatitis syndromes that were classified as of unknown origin. These findings however are preliminary and they need to be substantiated by others. The vesiviruses have also been shown to infect primates naturally (398) and experimentally (395). An African green monkey developed fever and vesicular lesions at injection sites 24 hrs after being inoculated with SMSV. Virus was recovered from lesions 96 hrs later and from the stool at 48 hrs post inoculation. Another SMSV type isolated from fish produced a disease in experimentally infected pigs identical to vesicular exanthema (397). Another vesivirus, was isolated from 3 dairy calves in a herd with persistent respiratory problems (394). The strain, designated Tillamook calicivirus, was not neutralized by 18 different calicivirus-typing sera available. The agent caused only minimal lesions in 2 experimentally exposed calves, but established a persistent infection with virus shedding for 45 days, when the experiment was terminated. Experimentally exposed swine to this virus developed clinical vesicular lesions. A vesivirus similar to the vesicular exanthema of swine calicivirus was detected in tissues from a naturally infected spontaneously aborted bovine fetus (400). A recent study on seroprevalence of vesiviruses in dairy and beef cattle in the U.S. (n=693) determined that 15.2% were positive by antibody ELISA using recombinant SMSV-5 peptide as antigen (233). Thus interspecies transmission and zoonosis should be under surveillance for all the calicivirus family members.
1.6 Immunity, treatment, prevention and control

1.6.1 Immune responses after natural and experimental infection

Because of the absence of an animal disease model, limited information on host-immunity to NoVs has been obtained from human volunteer studies. To date, correlates of protective immunity in adult volunteers (previously exposed) to NoV and in human recovered from NoV disease are not completely understood. In addition, immunity to NoV in children may differ from that in adults (136).

Volunteer studies included a cross-challenge between Norwalk virus (NoV GI) and Montgomery County (NoV GI) or Hawaii (NoV GII) strains (468). This study demonstrated that antigenically distinct NoVs exist and began to define the relationship of preexisting immunity with subsequent infection. In these studies, homologous Hawaii virus challenge 6 weeks later resulted in protection but heterologous (Norwalk virus then Hawaii virus) challenge was not totally effective in preventing symptoms with 3 of 6 individuals showing illness. Early studies in which the immune status of volunteers was tested, revealed that preexisting serum antibody to Norwalk virus was not associated with protective immunity, and that, paradoxically, persons with higher levels of preexisting antibodies were more likely to experience symptomatic disease (207). When serum and local jejunum antibodies were measured before Norwalk virus inoculation, no correlation between antibody titers and resistance were observed. The opposite was concluded from another study indicating that local jejunum and serum Norwalk-specific antibodies were significantly lower in resistant volunteers (33). The investigators further suggested that multiple exposures to NoV were needed to induce immunity to disease in some individuals (207). This suggests that the high antibody titers to Norwalk virus in adults in
developed countries, is an indicator of past exposure and susceptibility, whereas absence or existence of low titers in adults signifies relative resistance to infection. In contrast, high antibody titers in children may be due to short-term immunity and recent exposure (280). So while high serum antibody titers to Norwalk virus in adults suggest susceptibility and lack of protection; in children they correlate with protection. Sapovirus-specific antibodies also have been correlated with resistance to illness in infants and young children (303, 304).

In another Norwalk virus volunteer study, serological responses of individuals did not correlate with virus infection; not all volunteers who were clinically ill seroconverted to Norwalk virus (176). Generally, 50% or more of the individuals in a Norwalk virus outbreak demonstrate an antibody response to Norwalk virus.

Susceptibility to repetitive NoV infections could be due to short-term immunity or to the existence of so many diverse serotypes or strains that it is unlikely that individuals have immunity to all of them (120).

Cuckor et al. (61) studied the role of IgM antibodies in Norwalk virus-infected volunteers using a specific radioimmunoassay and concluded IgM antibodies peaked at the second week after inoculation and were still detectable at PID 21 and that IgM was not indicative of primary infection. When rechallenged 1-2 years later, volunteers who had been ill and produced an IgM antibody response suffered illness again and developed a secondary IgM antibody response of higher magnitude. This did not occur when challenge was performed 1-3 months after the first inoculation. Norwalk virus-specific IgM responses were not associated with subclinical illness.
Fecal IgA antibody responses to Norwalk virus were studied by inoculating volunteers with Norwalk virus VLPs first and then repeatedly challenging them with Norwalk virus (325). Volunteers who showed clinical symptoms had significantly higher prechallenge Norwalk virus-specific fecal IgA geometric mean titers and similarly when comparing infected versus noninfected individuals. Thus, preexisting Norwalk virus-specific fecal IgA was not protective and may be indicative of Norwalk virus susceptibility.

Salivary IgA and IgG antibodies to Norwalk virus were measured and compared to serum values in a volunteer study of 38 subjects inoculated with Norwalk virus (295). Infection was defined in 18 subjects by the shedding virus in stools or by serum IgG antibody seroconversion. Fifteen of these individuals had a ≥ 4-fold increase in salivary IgA antibody and the same number of responders was observed when salivary IgG antibody was measured. One volunteer who was uninfected had a Norwalk virus-specific salivary IgG antibody response. When this test was applied to a school outbreak, strain-specific salivary antibodies were detected by a 4-fold increase in salivary IgA antibodies in 67% of the cases with confirmed infection.

In a recent volunteer study (n=15) cellular and humoral immune responses to Snow Mountain virus (NoV GII/2) were evaluated by analyzing stool, serum, saliva antibodies, as well as peripheral blood mononuclear cell responses pre and post-inoculation (247). Sixty percent of volunteers became infected and had a 4-fold increase in serum IgG antibody titers to Snow Mountain virus. This specific serum IgG antibodies and to a lesser extent the salivary IgA antibody as well as the the Snow Mountain virus-activated T cells were cross-reactive with Hawaii virus (GII/1) but not
with Norwalk virus (GI/1). By post inoculation day (PID) 2 IFN-γ and interleukin (IL)-2, but not IL-6 or IL-10 were significantly increased in serum. It was highly probable that most volunteers in both the infected and control groups had been previously exposed to NoVs as peripheral blood mononuclear cells (mainly CD4+) from both groups secreted IFN-γ and other Th1 cytokines when stimulated with GII NoV VLPs (Snow Mountain virus and Hawaii virus VLPs, but not with Norwalk virus VLPs).

Reports on homologous and heterologous responses to NoV in outbreaks are numerous. While some authors concluded that responses were broadly reactive only within but not between genogroups (158), others observed that reactivity within the genogroup was stronger but, reactivity between genogroups also existed (95). For example, one of the volunteers inoculated with Hawaii (GII/1) virus developed a seroresponse to Norwalk virus (GI/1) (263). This is consistent with the fact that common epitopes exist (see section 1.3.7). Other investigators reported that responses to a GI strain can react with viruses that vary as much as 38% in amino acid sequence whereas responses to GII strains only reacted with strains that had 6.5% or less amino acid divergence (316). In another study, a panel of 7 NoV VLPs were used to evaluate the immune responses in 400 individuals involved in 37 NoV outbreaks. Most of patients seroconverted with IgG antibodies reacting to antigen homologous to the outbreak strain. However, they seroconverted in response to other genetically distinct antigens as well, suggesting no clear pattern of type-specific immune response (24). Although not widely used because of the difficulty of human trials, immune responses can determine serotypes. For example, VLPs of SaV Houston86 and Houston90 are antigenically distinct but share a common epitope(s) (204).
1.6.2 Strategies for prevention of environmental contamination by NoVs

Prevention strategies are based on 1) surveillance of possible infection sources such as contaminated shellfish and water; 2) regulation of oyster production and good food-handling practices and outbreak management (as sick leave required for ill personnel); and 3) decontamination procedures once an outbreak is underway to limit its spread geographically and in time (136).

The improvements in RT-PCR methods for viral detection from environmental samples have revealed the importance of these sources of infection in NoV epidemiology (131, 383, 470). Several reports on environmental contamination include information on the varied surfaces that may contribute to virus persistence in the areas surrounding an infected individual such as elevator buttons, taps, door handles, telephone receivers, curtains, lockers, carpets, toilets, tables, etc. (21, 53, 89, 132, 234, 467). These reports also indicate the importance of cleanup strategies with products that are effective in controlling NoVs, such as high concentrations of hypochlorite and the isolation of infected individuals (both patients and health care staff), because otherwise the outbreaks are extended. The persistence in the environment and transfer from surfaces to food of Norwalk virus and FCV showed that contaminated surfaces such as stainless steel, formica and ceramic could retain virus and transfer it to lettuce for up to 7 days. FCV was still infectious but there was a $7\log_{10}$ drop in virus titer whereas this could not be determined for Norwalk virus (82). A study consisting of the transfer of NoV from contaminated fecal material to hands and cloths and to other hand-contact surfaces showed that contaminated fingers could sequentially transfer virus to up to seven clean surfaces (21). This study also detailed a good cleaning procedure. Detergent-based
cleaning with a cloth to produce a visibly clean surface failed to eliminate NoV contamination. A combined 5000 ppm hypochlorite/detergent formulation was sufficient to decontaminate surfaces after secondary transfer, demonstrated using 1:10-1:80 NoV dilutions. Only this procedure produced a significant virus reduction when cleaning infected soiled feces, with NoV still being detected on up to 28% of these surfaces. When other surfaces were wiped with the used cleaning cloth, the virus was spread further. To reduce the risk of NoV persistence it was necessary to wipe the surface clean using a cloth soaked in detergent before applying the combined hypochlorite/detergent. The chlorine concentration found in the drinking water distribution system (3.75-6.25 mg/l) is inadequate to inactivate NoVs. Thus when a water supply has been contaminated with NoVs a concentration of 10 mg/l chlorine treatment is used (222).

In summary, it is important, due to the fast spread of the disease as well as the high attack rate, that the outbreak is recognized as soon as possible in order to limit the number of cases. This should be done by developing a case definition as well as keeping records of cases as part of a surveillance program so that control measures can be taken including movement restriction and temporarily closing facilities (262).

1.6.3 Antivirals

Currently there is no specific treatment for NoV gastroenteritis and only palliative rehydration is recommended. Research on antiviral drugs is greatly needed.

Interferon alpha has antiviral activity against FCV infection of cell cultures (294) by inducing the feline Mx gene 24 hrs post-IFN application (17). The Mx antiviral function is well known although its mechanism is unclear. Because of its GTPase
activity, Mx may regulate trafficking or transcription of the viral ribonucleoprotein complexes (244). It was established that IFNs also play a major role in MNV-1 when infecting mice and the SaV PEC/Cowden strain infection of cell cultures (see sections 1.5.1.4 and 1.5.8.1) (48, 215) which might also apply to other NoV and SaV members. Treatment with IFNs could be applied in case of an outbreak therapeutically and as a prevention drug for second contacts and the health care staff.

Antisense technology using phosphorodiamidate morpholino oligomers (PMO) is a promising approach to overcome the current diagnostic and therapeutic problems inherent with newly emerging viral diseases (393). This strategy, also known as RNA silencing is based on the principle that double stranded RNA is degraded in eukaryotic cells as a host defense mechanism and it is also refered to as RNA interference by its physical interference with translation. In an extensive review, Arora et al. (13) describe the various methods of delivering PMO into cells and animal models. Despite numerous reports of efficient intake of PMO by primary cell cultures and animal models, many have described that unassisted delivery is poor in most cell lines. Therefore, physical methods such as scrape-loading, syringe-loading and osmotic loading (116) or charged based methods such as cationic lipid complexes and arginine-rich peptide conjugation (310) delivery strategies have been developed for cell lines. However, primary cultures are more capable of taking up the PMOs without any assisted delivery similar to the in vivo situation (13). There are numerous studies where PMOs were administered locally (intra-tumor), parenterally, orally, intravenously, intraperitoneally or transdermally in various animal models such as rats, mice, rabbits, pigs, fish and frogs for a number of different pathologies including cancer and infectious diseases (13). There are also PMOs
that are actually being tested in human trials. The PMO bioavailability and distribution can be tested qualitatively by fluorescence microphotography and Western immunoblot or quantitatively by HPLC analysis. The PMOs are stable in plasma but are sensitive to degradation after prolonged exposure to low pH. In addition, Arora et al. (13) described the correlation of PMO activity \textit{in vitro} and \textit{in vivo} for dose and efficacy.

Antisense oligomers targeting one of the three ORF of caliciviruses of the \textit{Vesivirus} genus significantly inhibited viral replication in tissue culture (409). Porcine kidney and African green monkey kidney cells were infected with vesivirus isolates SMSV-13 and primate calicivirus PAN-1. The PMO with sequence complementary to the AUG translation initiation site of ORF1, ORF2, and ORF3 were evaluated for their effects on viral titers. The PMO were delivered by scrape-load to 50%-70% of the cells of the two cell lines, as measured by fluorescence microscopy and flow cytometry. Various PMO sequences antisense to an upstream region of ORF1 were effective in reducing viral titers up to 80% in a dose-dependent and sequence-specific manner. A PMO targeting ORF2, a scrambled PMO control sequence, and an unrelated PMO antisense sequence did not alter viral titers, whereas, a PMO targeting ORF3 caused a significant increase in viral titers. The extent of viral titer reduction by the PMO targeting ORF1 was proportional to the delivery of PMO to cells. These observations demonstrate that antisense PMO can disrupt caliciviral gene function in a nucleic acid sequence-specific manner and are potentially effective antiviral agents.

A study showed that histones can bind strongly to Norwalk virus VLPs suggesting that these could be used for antiviral purposes by preventing viral attachment to the target cell receptors (420). The advantage of these virus-binding molecules is that they are
non-toxic. Histone H1, interacts with Norwalk virus particles and also with the cell surface. Lactoferrins may act in a similar fashion as described for FCV in cell culture by the addition of bovine lactoferrin and lactoferricin B (285).

The development of infectious clones for both NoV and SaV as well as the SaV PEC/Cowden and MNV-1 tissue culture-adapted systems provide the opportunity to test antiviral drugs by detecting molecules that block or interfere with viral binding or replication (see section 1.5.8.1.)

1.6.4 Vaccines

Natural infection and repeated exposures to Norwalk virus induce at least short-term protective immunity (87, 280) suggesting that a vaccination approach to control human NoV infection is feasible. However, because of the antigenic diversity of human NoVs, an effective vaccine must protect against most, if not all, major circulating strains. Thus, vaccinating the general population may not be efficacious or cost-effective. However, certain populations at risk (e.g., military troops, the elderly in nursing homes, children, travelers, immunocompromised individuals) may benefit from a vaccine capable of inducing at least short-term immunity (87, 279). Several unique properties could contribute to the immunogenicity of NoV VLPs: VLPs are stable at low pH and are particulate, possibly targeting Peyer’s patches in the gastrointestinal tract. Thus, NoV VLPs are a potential candidate vaccine to prevent NoV infections (87).

The Norwalk virus (GI/1) and Dijon171 virus (GII/4) VLPs were immunogenic when inoculated orally or intranasally with or without addition of adjuvant into experimental mice (19, 147, 311, 344). Intranasal delivery of Norwalk VLPs was more
efficient in producing serum IgG and fecal IgA antibodies (19, 147, 311, 344) which were detected in both groups with or without cholera toxin (CT) or mutant labile toxin (mLT R192G) adjuvants (19, 147). However, the use of adjuvant induced higher titers of serum IgG antibodies, and enhanced the magnitude and duration of the immune responses, but the number of responders was not increased significantly. In most mice, serum IgG and fecal IgA antibody responses were detected by PID 9 and 24, respectively. In the absence of CT adjuvant, a Th1 response was dominant. The addition of CT shifted the response to a Th2 response in BALB/c mice, but not in CD1 mice (311, 344). When detoxified cholera toxin CT-E29H was delivered concomitantly with Norwalk VLP to BALB/c mice by mucosal routes, a low dose (5 μg) of vaccine with adjuvant delivered intranasally was more efficient than a higher dose (200 μg) delivered orally at inducing both cellular and VLP-specific IgG and IgA antibody responses (344). The use of adjuvant induced more antigen-specific IgA antibody secreting cells in the Peyer's patches as well as an increase in antigen-specific cells producing IL-4. Oral delivery resulted in more specific CD4+ and CD8+ T cells in Peyer’s patches and spleen and the addition of CT-E29H resulted in an increase of VLP-specific CD4+ cell populations in Peyer’s patches and both CD4+ and CD8+ populations in the spleen.
Similar results were obtained with Dijon171 GII/4 VLPs (311). In addition, in that study the cytokine profiles in cells from different lymphoid tissues (mesenteric and cervical lymph nodes, spleen, and Peyer’s patches) was determined by *in vitro* stimulation. A Th1/Th2-like response was observed in cervical lymph nodes and Peyer's patch cell cultures from mice inoculated by either route and with either adjuvant. Of interest, virus-specific T lymphocytes were present in the intestine after intranasal immunization.

The safety and immunogenicity of Norwalk virus VLPs as an oral immunogen have been evaluated in a phase I trial in healthy adult human volunteers (18). An important difference from the mice studies was that the human subjects had pre-existing serum virus antibodies to Norwalk virus. Oral administration of 100- and 250-μg doses of Norwalk virus VLPs boosted antibody responses in serum. Serum IgG and IgA Norwalk virus VLP specific antibodies were monitored by ELISA. Moderate rises in antibody titers were detected 2 weeks after vaccine administration and although the percentage of responders was similar to after natural infection, the magnitude of the antibody responses was lower than that induced after natural Norwalk virus infections. In addition the type of immune response was evaluated where both the oral vaccine and the natural infection stimulated predominantly Th2 responses (IgG1) (87). Similar findings were observed in BALB/c mice that were immunized orally with Norwalk virus VLPs with CT adjuvant (19) but not when the VLPs administered without adjuvant, where the response was mainly Th1.

Another vaccine delivery approach is the use a Venezuelan equine encephalitis (VEE) replicon expression system containing the Norwalk virus capsid gene cloned into
this vector and expressed in mammalian cells (171). The alphavirus genome in this system contains an RNA with the 5’ end coding for the nonstructural proteins and the 3’ end coding for the viral structural proteins under the control of a 26S promotor that drives transcription of the downstream sequences (gene of interest) as a subgenomic mRNA. Transfection of baby hamster kidney (BHK) cells with VEE replicon and a helper RNA encoding the VEE structural proteins that had been replaced by the Norwalk virus capsid gene resulted in VEE replicon particles (VRPs). Subcutaneous inoculation of mice with the VRPs expressing the Norwalk virus capsid proteins that self-assembled into high quantities of VLPs elicited specific systemic and mucosal immune responses to Norwalk VLPs. A mutated clone (3 aminoacid substitutions) that resulted in failure of the VLP to assemble induced a much lower mucosal and systemic immune response indicative of the importance of the particle integrity. Serum antibody responses to the VRP-NV replicon using this delivery system was far superior to oral inoculation of mice with VLPs prepared from the VEE-Norwalk virus replicon construct but delivered like current models for NoVs vaccination.

Transgenic plants represent a novel and potentially economical system for production of vaccine antigens and edible plants themselves can serve as oral delivery systems (416).

Norwalk virus capsid protein expressed in tobacco leaves, tomatoes and potatoes stimulated serum and mucosal antibody responses in most mice in the presence or absence of CT (275), but responses were lower than after oral inoculation of VLPs.

Potato tubers containing Norwalk virus VLP were also immunogenic when given to human volunteers (417). Among 20 volunteers, 19 (95%) volunteers developed
significant increases in the numbers of IgA antibody-secreting cells in whole blood, 4 (20%) developed Norwalk virus IgG antibodies in serum, but only 6 (30%) developed Norwalk virus-specific fecal IgA antibodies. The protective efficiency of these antibodies was not evaluated. A study using virus challenge to evaluate the protection rates, was performed in rabbits vaccinated and challenged with RHDV (272). The rabbits were fed the capsid protein VP60 (the equivalent of VP1 in NoV) of RHDV in transgenic tubers of potato in lyophilized tuber homogenates in 100 or 500 μg doses administered on days 0, 21, 42 and 63. Only 4 of 5 rabbits in the high dose group but none of the 5 rabbits in the low dose group developed an immune response that was measured by antibody ELISA using the VP60 antigen. After challenge with virulent RHDV only one rabbit with the highest antibody titer was partially protected and survived. Controls and rabbits in the low dose group died within 48 hrs of challenge whereas rabbits in the high dose group perished 24 hrs later, suggesting partial protection.

Whether the induced antibody responses by the Norwalk virus VLP are protective is unknown. Neutralizing antibodies that are often critical for protective immunity could not be monitored because there is no cell culture system for human NoVs and challenge studies are difficult to perform. Therefore, the extrapolation from animal model vaccination studies such as for porcine SaV and NoV and bovine NoV are extremely valuable in addition to their application to the animal industry. The expression of bovine NoV VLPs (162) has permitted the evaluation of VLPs as potential vaccines followed by challenge studies to evaluate protection (160). Gnotobiotic calves were vaccinated with two or three doses of VLPs (250ug) coadministered with oil, mLT (R192G) or immunostimulating complexes (ISCOM). Only the group of calves that was vaccinated
intranasally with VLP+mLT developed detectable VLP-specific fecal IgA antibodies that provided partial protection upon challenge with virulent bovine NoV. Diarrhea onset was delayed and shortened to 1-2 days compared to 2 to 6 days in the other vaccinated groups of VLP with oil or ISCOM and 8 to 9 days in controls. Neither vaccine strategy protected against viral shedding measured by antigen ELISA. All convalescent calves had high titers of fecal IgA and were completely protected upon rechallenge.

Another area to explore is the use of DNA vaccines. A DNA vaccine constructed from a plasmid vector carrying the FCV capsid gene was inoculated in 3 doses of 100 μg 2 weeks apart by intramuscular injection (404). No antibody responses could be detected before challenge with virulent FCV, but post-challenge, vaccinated cats had higher antibody titers than control cats. Both vaccinated and control cats showed clinical signs although they were milder in the vaccinated group, suggesting partial protection.
1.7 REFERENCES


from a single, functionally bicistronic, subgenomic mRNA. J Gen Virol 77 (Pt 1):123-7.


251. **Liu, B. L., I. N. Clarke, E. O. Caul, and P. R. Lambden.** 1995. Human enteric caliciviruses have a unique genome structure and are distinct from the Norwalk-like viruses. Arch Virol 140:1345-56.


against feline calicivirus infection using a plasmid encoding the mature capsid protein. Vaccine 20:1787-96.


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Table 1.1 List of most frequently used primers for human NoV detection
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Table 1.2 List of existing Virus-like particles for human and animal NoV and SaV.

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Table 1.3 Norovirus and Sapovirus-specific monoclonal antibodies
Figure 1.1 Immune electron micrograph of 1) Norovirus 2) Sapovirus. Bars=50 nm.
From www.virology.net/Big_Virology/BVRNAcalici.html F. P. Williams
Figure 1.2 Unrooted tree of the capsid (VP1) amino acid sequence. The depicted tree shows calicivirus genera (boxes) and genogroups (bold) obtained as a consensus of trees from 1000 bootstrapped data sets analyzed using UPGMA algorithm (MEGA 2.1). Genbank accession #: Norwalk virus (NV) M87661, Southampton virus L07418, Desert Shield virus (DSV) U04469, Hawaii virus (HV) U07611, Snow Mountain virus (SMV) AY134748, Toronto virus (TV) U02030, Arg320 AF1900817, Lordsdale virus (LV) X86557, Alphatron AF195847, Manchester virus X86560, all of human origin. The SW43 AB074892, QW48 AY823303, QW101 AY823304, QW218 AY823307, Cowden AF182760 all of swine origin; Newbury agent-2 (NA-2) AF097917, Jena AJ011099, CV186 AF542084, Nebraska virus (NB) AY082891, all of bovine origin. Murine Norovirus-1 (MNV-1) AY228235, San Miguel sea lion virus-1 (SMSV-1) AF181081, Primate calicivirus-1 (PAN-1) AF091736 and Rabbit Hemorrhagic Disease virus (RHDV) AY928270.
Figure 1.2 Phylogenetic analysis of the complete capsid amino acid sequence of Norovirus strains (from D. Zheng et al., 2005, reference #475). Genogroups are circled and genoclusters are boxed.
Figure 1.3 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) Subgenomic RNA (adapted from Green et al., 2000, reference #136). Boxes contain coding sequences for the for N-terminal protein (N-term), NTPase, p20, VPg, Protease (Pro), RNA dependent RNA polymerase (RdRp), capsid protein (VP1) and small basic protein (VP2). Numbers indicate nucleotide positions.
Figure 1.4 Ribbon representation of the structure of Norwalk virus recombinant capsid protein (from Prasad et al., 1999, reference #352) and cartoon representation of the surface structures of recombinant Norwalk virus (a), Grimsby virus (b), Parkville virus (c), San Miguel sea lion virus (d), respectively (from Chen et al., 2004, reference #54).
Figure 1.5 Epidemiological data of modes of transmission (adapted from Fankhauser et al., 1998, reference #93) and most common source settings (adapted from Glass et al., 2000, reference #120).
Figure 1.6 Diagram of major biosynthetic pathways of the ABO(H), Lewis and related antigens (adapted from Marionneau et al., 2001. Reference #264). Antigens are in boxes; enzymes required to catalyze each step are in *italics.*
CHAPTER 2

PATHOGENESIS OF A GENOGROUP II HUMAN NOROVIRUS IN GNOTOBIOTIC PIGS

2.1 SUMMARY

We evaluated the gnotobiotic (Gn) pig as a model to study the pathogenesis of human Norovirus (HuNoV) and to determine the target cells for viral replication. Sixty-five Gn pigs were inoculated with fecal filtrates of NoV/GII/4/HS66/2001/US strain or with pig passaged intestinal contents (IC), and euthanized acutely (n=43) or after convalescence (n=22). Age-matched Gn piglets (n=14) served as mock-inoculated controls. Seventy four-percent (48/65) of the inoculated animals developed mild diarrhea compared to 0/14 controls. Pigs from post-inoculation days (PID) 1-4 tested positive for HuNoV by RT-PCR of rectal swabs fluids (29/65) and intestinal contents (IC) (9/43) by antigen ELISA using antiserum to virus-like particles (VLP) of HuNoV GII/4. No control pigs were positive. Histopathologic examination showed mild lesions in the proximal small intestine of only one pig (1/7). Seroconversion after PID 21 was detected by antibody ELISA in 13 of 22 virus-inoculated pigs (titers 1:20-1:200), but not in controls.
Immunofluorescent microscopy using a monoclonal antibody to HuNoV GII capsid revealed patchy infection of duodenal and jejunal enterocytes of HuNoV-inoculated pigs (18 of 31) with a few stained cells in ileum and no immunofluorescence in mock-inoculated controls. Immunofluorescent detection of the viral RNA-dependent RNA polymerase antigen in enterocytes confirmed replication. Transmission electron microscopy of intestine from HuNoV-inoculated pigs showed disrupted enterocytes, with cytoplasmic membrane vesicles containing calicivirus-like particles of 25-40 nm in diameter. In summary serial passage of HuNoV in pigs, with occurrence of mild diarrhea and shedding, and immunofluorescent detection of the HuNoV structural and nonstructural proteins in enterocytes confirm HuNoV replication in Gn pigs.

2.2 INTRODUCTION

Human Noroviruses (HuNoVs) are a major cause of foodborne gastroenteritis worldwide. Because they do not grow in cell culture and there is no animal model for HuNoVs, pathogenesis studies have been hampered. Thus, little is known about their replication strategies or induction of neutralizing antibodies.

The limited information on their pathogenesis is from human volunteer studies of HuNoV infections in which villus atrophy in duodenal biopsies and presence of malabsorptive diarrhea were described (1, 7, 8). No information is available on lesions in other portions of the intestine of these volunteers (9). Intestinal transplant pediatric patients that were diagnosed with HuNoV infection developed secretory or osmotic diarrhea (18, 19, 30). These patients had prolonged diarrhea (17-326 days) due to immunosuppressive therapy. The detection of HuNoV RNA and the clinical symptoms
remitted after reduction of the immunosuppressive therapy. Usually in exposed individuals, histologic lesions correlate with diarrhea, but in one report, lesions in volunteers who did not show clinical symptoms were described (40). There are also numerous reports of asymptomatic individuals who were infected with HuNoVs and shed virus in the feces (11, 27).

Most past attempts to study these viruses in an animal model may have failed because: (i) the human strains that were used were not closely related to the host animal NoV strains; (ii) sensitive detection techniques were lacking; and finally (iii) the role of histo-blood group antigen (HBGA) phenotypes in differential susceptibility of the host was unrecognized. Our goal was to adapt a HuNoV strain to replicate in the Gn pig to develop an animal model for the study of HuNoV pathogenesis. Gnotobiotic pigs are good models for human enteric diseases (38) because pigs resemble humans in their gastrointestinal anatomy, physiology and immune responses. The Gn pigs are immunocompetent at birth, but they lack maternal antibodies and previous or ongoing exposure to microbial agents, including caliciviruses.

Recently viral RNA genetically similar to that of human NoV GII (65-71% amino acid sequence identity in the capsid gene) was detected in pigs in Japan (44, 45) and Europe (21, 46). In U.S. swine our laboratory detected both viral RNA and virus particles similar to GII HuNoV (70% sequence identity in the capsid region) which were infectious for Gn pigs (48). Our approach to infect Gn pigs with a HuNoV was to use a GII strain that is closely related genetically to the identified GII porcine NoVs and that has a broad HBGA binding pattern because little information or reagents are available for pig HBGA. Additionally we used sensitive assays and reagents including RT-PCR to
detect fecal shedding, virus-like particles (VLPs) for serological assays and antisera to these VLPs for antigen ELISA to increase the sensitivity of virus detection in the exposed Gn pigs.

In this study Gn pigs were inoculated orally (n=63) or intravenously (n=2) with a HuNoV GII/4 strain and infection was monitored by the presence of diarrhea, fecal virus shedding, infected cells in intestinal tissues and seroconversion. Age-matched mock-inoculated Gn pigs (n=14) served as controls. Most virus-inoculated pigs developed diarrhea and nearly half shed virus in the feces or seroconverted. In addition, viral antigen and calicivirus-like particles were detected in the cytoplasm of intestinal epithelial cells of some inoculated Gn pigs by immunofluorescent staining to the capsid and RNA dependent RNA polymerase (RdRp) antigens and electron microscopy, respectively. This data suggests that HuNoV replicated in intestinal epithelial cells and was shed in feces of at least some virus-inoculated pigs.

2.3 MATERIALS AND METHODS

2.3.1 Experimental animals and inocula

Gnotobiotic pigs were delivered and maintained as previously described (28). All animal protocols used in this study were approved by the Institutional Laboratory Animal Care and Use Committee (ILACUC). In a preliminary pilot study, 4 human NoV (2 of GI and 2 of GII) and 1 human SaV strains were tested in Gn pigs. The results of our pilot studies are summarized in (Table 2.1). Although all strains showed promising results; we
selected the NoV GII/4 strain to continue the pathogenesis study based on the preliminary results, the impact of this strain on disease in the human population and the availability of large amount of fecal sample.

The original human fecal sample was designated NoV/GII/4/HS66/2001/US (HS66) strain, and was collected from a child with diarrhea at Children’s Hospital in Columbus, Ohio and kindly provided by Dr. J. Hughes, The Ohio State University. We used the inoculum as a single aliquoted pool throughout our study. The viral titer of this sample was semiquantified, by real-time RT-PCR (Cheetham, Souza and Saif, unpublished), as approximately 5.4 ×10⁶ particles/ml (assuming that 1 viral genome = 1 viral particle). The original human fecal sample was designated passage 0 (P0). Passage 1 (P1) was an RT-PCR positive intestinal content (IC) pool from Gn pigs inoculated with P0 and passage 2 (P2) was RT-PCR positive intestinal contents (IC) of a Gn pig inoculated with P1. Two additional pigs were orally inoculated with a fecal filtrate of P0-inactivated virus prepared by incubation with 0.01M binary ethylenimine (BEI) for 18 hrs at 37°C with continuous agitation as previously described (53). Sodium thiosulphate (1M) was added to a final concentration of 10% to inactivate the BEI followed by dialysis with a cassette (Pierce Biotechnology, Rockford, IL). Sixty-five Gn piglets were inoculated with 5 ml of a 1:10 dilution in minimal essential medium (MEM) (Gibco, invitrogen, Carlsbad, CA) of HS66 strain from the original human fecal sample or derivatives from it after serial passages in Gn pigs. Pigs were divided into groups according to the inocula (P0, P1, P2). Group A pigs (n=37) were orally inoculated with P0 except for one pig that was inoculated intravenously. Group B pigs (n=18) were orally
inoculated with P1 except for one pig that was inoculated intravenously. Group C pigs (n=10) were orally inoculated with P2. Fourteen age-matched piglets were orally inoculated with MEM and served as mock-inoculated controls. Twelve of these pigs (6 from group A, 3 from group B and 3 controls) received oral dexamethasone (1mg/kg/day) for 10 days to mimic the immunosuppression in transplanted individuals (18, 19, 30).

Daily rectal swabs were collected from 0-10 post inoculation days (PID) and diarrhea was assessed (scores 0=normal; 1=creamy; 2=pasty; 3=watery). Samples with scores 2 and 3 were considered to have diarrhea. Two researchers scored the samples from all the Gn pigs. Pigs were euthanized during the acute phase (PID 1-5) (n=51, 43 virus-inoculated and 8 controls) and intestinal tissues were harvested for histopathologic examination (n=9), immunofluorescent microscopy (n=39) and transmission electron microscopy (TEM) (n=13). A subset of 10 pigs (8 virus-inoculated and 2 controls), were bled daily for 10 days to evaluate the presence of viremia. Some pigs (n=28, 22 virus-inoculated and 6 controls) were kept until PID 21 to evaluate seroconversion and the duration of viral shedding, diarrhea.

2.3.2 RT-PCR, internal control and real time RT-PCR and microwell hybridization

The RNA extraction from rectal swab fluids, 1:20 dilutions of IC and 1:50 dilutions of serum were performed using TRIZOL® LS (Invitrogen) as directed by the manufacturer. A one-step RT-PCR assay was conducted using the Mon 431/433 primers (34) directed to the RdRp region of HuNoV GII. The RT-PCR conditions were 42°C for
1 h, 94°C for 3 minutes and 40 cycles of 94°C for 30 sec, 50°C for 30 sec and 72°C for 30 sec, with a final extension of 72°C for 10 minutes. Amplicons were visualized by electrophoresis in 2% agarose gels stained with ethidium bromide under UV light. The product specificity was confirmed by sequencing or microplate hybridization as described by Wang et al (47) using a probe specific for the HS66 strain (PmonHS66 5’CTTGCTAATTTGCTGTAATGATGGGCCGTGGA-3’). Negative controls for RNA extraction and RT-PCR assays (mock-inoculated pigs and water) were included in each assay. To detect RT-PCR inhibitors and overcome possible false negative results often encountered with fecal samples, we engineered a competitive internal control. Briefly, sequences complementary to primer pairs P289/290 (17) and Mon 431/433 were added by RT-PCR to a β-2 microglobulin DNA sequence at the 3’ and 5’ ends and subsequently cloned into a plasmid (pCR2.1 vector, Invitrogen). This plasmid served as template for in vitro transcription (Promega, Madison, WI) of the internal control RNA. The specific HuNoV product had a size of 211bp, whereas the internal control was 320bp long. The RNA samples that showed inhibition were diluted in DEPC treated water and retested as described previously (47).

One-step real-time RT-PCR was standardized using serial dilutions of known concentration of the internal control (described above). The internal control was used as an external amplicon to generate a non-competitive standard curve with Mon 431/433 primers using SYBR Green I (Roche, Indianapolis, IN) (Cheetham, Souza and Saif, unpublished) with the same cycling conditions as described earlier for viral shedding detection.
2.3.3 Cloning and expression of HS66 capsid gene and production of VLPs

Enzyme-linked immunosorbent assays (ELISA) were initially standardized using VLPs and hyperimmune antisera to a strain (MD145, GII/4) from the same genotype as HS66, kindly provided by Dr. K Green (NIAID, NIH, Bethesda, MD). Subsequently, we cloned the capsid gene of HS66 and generated a recombinant baculovirus clone expressing the HS66 capsid gene. A baculovirus expression system was used to produce HS66 NoV VLPs using Spodoptera frugiperda (Sf9) insect cells (16). First, the capsid gene was amplified by RT-PCR from the original fecal sample RNA using forward primer HS66ORF2f 5’GGCTCCCAGTTTTG TGAATG3’ and reverse primer HS66ORF2r 5’AACCAAGTCCAGAGCCAAGG3’ with the following reaction characteristics: annealing temperature of 52°C and extension time of 2 minutes. The amplicon was first cloned into a pCR2.1 vector (Invitrogen) and then subcloned into a baculovirus transfer vector, pBlueBac4.5 (Invitrogen) using the EcoRI restriction enzyme site. The recombinant plasmid that contained the full-length capsid gene in the correct orientation and the linearized wild-type baculovirus DNA were used to cotransfect Sf9 cells. After confirming the recombinant plaques by PCR and 3 rounds of plaque purification, we produced a recombinant baculovirus stock containing the capsid gene. For VLP production, Sf9 cells were infected with the recombinant baculovirus at a multiplicity of infection (MOI) of 10 and harvested at PID 7-10. The supernatants were collected and centrifuged at 3,000 X g for 30 minutes to remove cells. With the VLPs still in the liquid phase, concentration was performed by ultracentrifugation at 110,000 X g for 2 hrs at 4 °C through a 40% sucrose cushion. The pellet containing the VLPs was resuspended in TNC buffer (10mM Tris HCl, 140mM NaCl, 10mM CaCl₂, pH 7.4) and
the VLPs were purified by CsCl gradient ultracentrifugation at 150,000 X g at 4°C for 18 hrs. The visible VLP bands were collected by aspiration, diluted in TNC buffer and repelleted by ultracentrifugation 110,000 X g at 4°C for 2 hrs to remove the CsCl. The self-assembled VLPs were tested for integrity and reactivity by immune electron microscopy (IEM) (Figure 2.1), antigen ELISA and Western blot (Figure 2.2). The negative stained IEM was performed as previously described. For the Western blot CsCl gradient purified VLPs were separated in 10% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis, blotted onto nitrocellulose membranes and incubated with guinea pig MD145 VLPs polyclonal antiserum (1:500) in PBS containing 5% nonfat dry milk and followed by goat anti-guinea pig IgG HRP conjugated. Forty percent sucrose cushion purified Sf9 insect cell proteins were used as negative control. The protein concentration was measured using the Bradford quantification method (Biorad, Hercules, CA).

2.3.4 Production of hyperimmune serum

A guinea pig was first immunized with HS66 VLPs (500 µg) mixed with Freund’s complete adjuvant via subcutaneous injection followed by 4 booster injections of the same dose in Freund’s incomplete adjuvant with a 10-day interval between doses. The IgG VLP-specific antibody ELISA titer of the hyperimmune antiserum was >10,000.
2.3.5 Antibody ELISA

The antibody ELISA was adapted from previously described procedures (15) with modifications. We used CsCl purified HuNoV GII/4 MD145 VLPs or HuNoV GII/4 HS66 VLPs to coat 96-well microtiter plates (Nalge Nunc, Rochester, NY) at a final concentration of 2.5 µg/ml (100µl/well) in coating buffer (0.05M carbonate buffer pH 9.6). Plates were incubated at 4°C overnight. Blocking was performed with 2% skim milk in PBS buffer (10mM potassium phosphate, 150 mM NaCl, pH7.4) at 37°C for 1 h.

Four-fold serial dilutions, beginning at 1:10, of the pig paired serum samples were added to wells and the plates were incubated for 3 hrs at 37°C. A mouse monoclonal antibody to pig IgG (3H7D7) conjugated to biotin was added to the plates (1:10,000) and plates were incubated for 1 h at 37°C, followed by addition of streptavidin-HRP (1:2000) and incubated at 37°C for 1 hr. The assay was developed with tetramethylbenzidine (TMB) (KPL, Gaithersburg, MD), incubated for 15-20 minutes at room temperature (Rt) and stopped with HCl (0.1M). Plates were washed 4 times between each step with PBS containing 0.5% Tween 20. Serum from mock-inoculated control pigs and unrelated rotavirus VLP (53) coated wells were used as negative controls. Positive samples were those with an absorbance equal or greater than the cutoff which was determined as the mean of the antibody-negative control wells plus 3 times the standard deviation. For the paired sera (preinoculation and PID 21), seroconversion was defined as a four-fold increase in the convalescent serum titer as compared to the preinoculation serum sample.

The immunostaining of fixed recombinant baculovirus-infected Sf9 cell assay was performed as previously described (54) as another way to detect HuNoV-specific
antibodies. Briefly, nearly confluent monolayers of Sf9 cells in 96 well plates were infected with the recombinant baculovirus expressing the HS66 capsid protein at an MOI of 5 and incubated at 27°C for 5 days. Medium was aspirated from the wells and the plates were then air dried and fixed with 3.7% formaldehyde for 30 minutes at Rt. Cells were permeabilized with 1% Triton X-100 (Sigma, St. Louis, MO) in TNC buffer for 5 minutes at Rt and then rinsed with PBS (pH 7.4). Serial two-fold dilutions of pig serum samples beginning at 1:10 were added to the wells and incubated for 2 hrs at 37°C. Plates were rinsed in PBS, goat anti-pig IgG antibody conjugated to HRP was added, the plates were incubated for 2 hr at 37°C and developed with 3-amino-9-ethylcarbazole (AEC) substrate (Vector Labs, Burlingame, CA). The antibody titer was defined as the reciprocal of the highest serum dilution at which a positive cell could be detected using light microscopy.

2.3.6 Antigen ELISA

The antigen ELISA was adapted from previously described procedures (15) with modifications. Briefly, plates were coated with the guinea pig hyperimmune antiserum to HS66 VLPs diluted 1:5000 in coating buffer and incubated at 4°C overnight. Blocking was performed as described for the antibody ELISA. Rectal swab fluids and serum were diluted 1:10 and IC were diluted 1:40 before addition to duplicate wells. The plates were incubated for 3 hrs at 37°C. Rectal swab fluids and IC samples from mock inoculated pigs and preinoculation rectal swab samples from HuNoV-inoculated pigs were used as negative controls along with wells coated with preinoculation guinea pig serum. The
original HS66 sample was used as a positive control. Rabbit anti-MD145 VLPs (1:3000) followed by goat anti-rabbit IgG conjugated to HRP (1:2000) were added and the plates were incubated for 1 h at 37°C. Plates were washed 4 times between each step with PBS containing 0.5 % Tween 20 (except after the rectal swab fluid/IC, which were washed 6 times). The assay was developed with TMB and incubated for 15-20 minutes at Rt and stopped with HCl (0.1M). Positive samples were those with an absorbance equal or greater than the cutoff which was determined as the mean of the negative control wells plus 3 times the standard deviation.

2.3.7 Fluorescent microscopy

Segments of duodenum, jejunum and ileum from inoculated pigs euthanized in the acute diarrhea phase (n=31), or samples from age-matched mock-inoculated pigs were collected (n=8). Indirect immunofluorescence was performed on paraffin embedded sectioned intestinal tissues or on whole mount samples.

2.3.7.1 Indirect immunofluorescence using paraffin embedded intestinal tissues

The procedure was modified from the protocol described by Shoup et al (41). Intestinal tissues were fixed in 10 % neutral formalin for 10-18 hrs, dehydrated in a graded ethanol series and embedded in paraffin. Five micron sections were cut and collected on positively charged glass slides (Fischer Scientific, PA). Slides were kept at 60°C for 20 minutes, deparaffinized in xylene twice for 5 minutes and rehydrated through
the graded ethanol series. To unmask the antigens, proteinase K (DAKO, Carpinteria, CA) treatment was applied to the slides for 3 minutes, then slides were washed in PBS and blocked with 1% normal goat serum for 20 minutes at Rt. A primary monoclonal antibody (MAb) NS14 (1:500) was incubated overnight at Rt. This MAb (20), kindly provided by Dr. M. K. Estes (Baylor College of Medicine, TX), has been mapped to an epitope in the C-terminal of the P1 domain of the capsid protein of all GII NoVs tested (32). The controls included HuNoV-infected pigs and mock-inoculated controls tested with a MAb to the spike protein of an unrelated virus (Transmissible Gastroenteritis Virus, TGEV, 25C9) (41); and age-matched, mock-inoculated pigs tested with MAb NS14. Samples were washed twice with PBS and the secondary antibody, goat anti-mouse F(ab')2 IgG labeled with AlexaFluor488 that produces green immunofluorescence (Invitrogen, A11075) was applied at a 1:400 dilution for 1 h at Rt. Cell nuclei were counterstained with propidium iodide that produces a red color (Invitrogen, P1304MP) following the manufacturer’s instructions.

2.3.7.2 Indirect immunofluorescence on whole intestinal tissue mounts

The intestinal tissues were fixed with 4% paraformaldehyde, 0.2% glutaraldehyde in 0.1M potassium phosphate buffer (PPB) pH 7.4 for 2 hrs at Rt, washed four times with PPB, and soaked with PPB containing 50mM glycine for 1h at Rt, or kept over night at 4°C. After permeabilization with 0.1% TritonX100 in PBS for 1 h at Rt, tissues were washed with PBS, blocked with PBS containing 2% bovine serum albumin (BSA), 5% normal goat serum for 30 minutes at Rt and incubated with MAb NS14 (1:500) (17, 26)
overnight at 4°C in the incubation buffer (10mM potassium phosphate buffer (pH7.4) containing 150 mM NaCl, 10mM NaAzide, 0.2% BSA). After six washes with PBS, incubation with a 1:600 dilution of the secondary antibody, AlexaFluor488 which produces a green color, labeled goat anti-mouse IgG (Invitrogen, A11075) in incubation buffer was performed overnight at 4°C. Samples were counterstained with the nuclear stain SYTOX Orange (Invitrogen, S11368) giving a red color and the actin stain AlexaFluor633 labeled phalloidin (Invitrogen A222884) producing a blue color. In double labeling experiments, we used the MAb NS14 for the viral capsid, and a guinea pig antiserum to the viral RdRp (r“2A” NIH# 60,000) from NoV GII/4 MD145 strain (kindly provided by Dr. K. Green, NIH) (1:500) as primary antibodies and the pre-hyperimmunization serum of the same guinea pig (NIH#59,478) as control. We also used a 1:600 dilution of the AlexaFluor488 labeled goat anti-mouse IgG (Invitrogen, A11075), and the AlexaFluor576/603 labeled goat anti-guinea pig F(ab')2 IgG (Invitrogen, A11017) providing a red color, as secondary antibodies. Cell nuclei were counterstained with TOPRO-3 iodide (642/661) dye (Invitrogen, A22284), that produces a blue color, following the manufacturer instructions. Samples were examined using a Leica TCS-SP laser scanning confocal microscope (Leica, Wetzlar, Germany).

2.3.8 Histologic examination

In addition to the intestinal tissues, pieces of lung, kidney, liver, spleen and mesenteric lymph nodes, were collected from 7 virus-inoculated and 2 control pigs. Tissues were fixed in 10% neutral formalin for five days, dehydrated in a graded ethanol
series, embedded in paraffin, cut in 5 micron sections and collected on glass slides (49). Deparaffinization was performed by placing the slides in xylene twice for 5 minutes with rehydration through the graded ethanol series before being stained with Mayer’s hematoxylin and eosin and examined microscopically.

2.3.9 Detection of Apoptosis

Apoptosis was examined in epithelial cells of small intestinal tissue sections (duodenum) of 5 HuNoV-inoculated pigs (2 pigs from group A, 1 pig from group B and 2 pigs from group C, none of which were treated with dexamethasone) and 5 mock-inoculated control pigs by terminal deoxynucleotidyl transferase (TdT) for the TUNEL reaction (TdT-mediated dUTP nick end labeling). This technique detects endonucleolysis by incorporating labeled nucleotides into DNA strand breaks. An In situ cell death detection kit (Roche) was used for paraffin embedded tissues as directed by the manufacturer and was developed with AEC substrate (Vector Labs). Quantification was performed by counting the total number of apoptotic cells on one field at a total magnification of 20 X. Positive (DNAse I treated) and negative (no TUNEL) slides were included each time the test was performed as indicated by the manufacturer.

2.3.10 Transmission Electron Microscopy

Segments of duodenum, jejunum and ileum from inoculated pigs euthanized in the acute phase (n=10) and confirmed positive for HuNoV by RT-PCR or mock-inoculated age-matched control pigs (n=3) were collected and transferred
immediately to the fixative; 3% glutaraldehyde, 1% paraformaldehyde in 0.1M PPB pH 7.4, then trimmed to 0.2 cm$^2$ pieces and fixation was continued for 2-3 hrs at Rt. After washing once with PPB and three times with distilled water, tissue samples were post-fixed with 1% osmium tetroxide, and 1% uranyl acetate in distilled water for 1 h at Rt, washed three times with distilled water, dehydrated in a graded ethanol-acetone series, and embedded in Spurr’s resin (EMS). For each pig, ten thin sections (80 nm) from 3 blocks at 3 different levels were cut using diamond knives, collected on 200 nm mesh copper grids and stained with 2% aqueous uranyl acetate and Raynold’s lead citrate stain. Samples were viewed using a Hitachi H-7500 (Hitachi, Tokyo, Japan) transmission electron microscope.

2.3.11 Statistical analysis

The Fischer’s exact test was used to evaluate the proportion of pigs with RT-PCR or antigen ELISA positive IC or positive immunofluorescence of the intestinal tissues at euthanasia (early: PID2-3 or late: PID 4-5), the proportion of pigs that seroconverted in each inoculum group and the proportion of pigs with dexamethasone treatment that showed diarrhea or had fecal virus shedding. Kruskal-Wallis test (non parametric) was used to compare the onset and duration of diarrhea and shedding in each inoculum group.
As no increase in severity or duration of diarrhea or fecal viral shedding was observed, the dexamethasone-treated pigs were analyzed together with the untreated pigs. Statistical significance was assessed at a $P$ value of <0.05. The data were analyzed using the Statistical Analysis Systems (SAS Institute Inc., Cary, NC).

2.4 RESULTS

The data on diarrhea and viral shedding (onset and duration), seroconversion and detection of infected cells in the intestinal tissues by IF, are summarized in Tables 2.2 and 2.3.

2.4.1 Presence of diarrhea, fecal viral shedding, viremia and seroconversion

Diarrhea was observed in 74% (48/65) of the inoculated pigs occurring from PID 1 (average onset PID 2) and persisting up to 5 PID (average 2 PID) (pigs included in Tables 2.2 and 2.3). Most pigs with clinical manifestations showed mild diarrhea (score=2), with only a few showing more severe diarrhea. Forty-four percent (29/65) of the inoculated pigs shed virus in feces detected by RT-PCR from PID 1 to a maximum of 4 days (average duration 3 days although some pigs shed for 1 day only). Shedding was detected for longer in some cases (up to 8 days) (data not shown) by amplicon hybridization which is more sensitive when compared to gel electrophoresis (47). Twenty-one percent of the IC from virus-inoculated pigs euthanized in the acute phase (PID 2-3 or 4-5, Table 2.2) were positive for viral antigen by Ag ELISA and 39% (17/43) for viral RNA by RT-PCR, respectively. In P0-inoculated pigs more IC were positive by RT-PCR and Ag ELISA when pigs were tested early (PID 2-3) ($P= 0.09$). There were
significant differences for duration of virus shedding between inoculum passages ($P=0.004$) with P0 inoculated, pigs having significantly longer viral shedding than P1 and P2 inoculated pigs (Table 2.3). Amplicons from the original human fecal samples and from the IC of Gn pigs were directly sequenced and sequenced after cloned (3 clones each). Sequences of the 200 bp products targeting the RdRp did not show any mutations or microheterogeneity.

From the 2 pigs inoculated by the intravenous route, only the one inoculated with P0 shed virus in feces whereas the pig inoculated with P1 did not, although both had mild signs of diarrhea. The Gn pigs treated with dexamethasone at the given dose and period of time did not differ statistically in diarrhea or virus shedding compared to HuNoV-inoculated non-dexamethasone treated pigs. The mock-inoculated Gn pigs treated with the same dexamethasone regimen did not develop diarrhea. Five of 8 P0-inoculated pigs tested had HuNoV RNA detected by RT-PCR in serum on PID 1 (n=3) or 2 (n=2); sera from the other 3 P0-inoculated and 2 mock-inoculated controls were negative for viral RNA during the 10 days tested (data not shown). Four of 5 viremia-positive pigs were also confirmed by antigen ELISA. Seroconversion occurred in 59% of the inoculated pigs (Table 2.3), independent of the HS66 pig passage number, but with low to moderate geometric mean serum IgG antibody titers (titer range 20-200, data not shown). None of the mock-inoculated control pigs developed antibodies to HuNoV. We confirmed the seroconversion results using the immunostaining assay for the HS66 capsid recombinant baculovirus-infected Sf9 cells.
2.4.2 Identification of infected cells by immunofluorescence (IF)

Confocal fluorescent microscopy was used to confirm HuNoV infection of the Gn pig small intestinal tissues and to characterize the infection at the cellular level. We performed IF localization using paraffin sections and whole mount small intestinal tissue samples. The first method allows the visualization of deeper portions of the small intestine and permits testing of archived samples in paraffin blocks whereas the whole mount tissue method may be more sensitive because there is no masking of the antigens by embedding but needs fresh tissues.

Immunofluorescence of whole mount small intestinal segments from virus-inoculated pigs showed that the distribution of positively (green) stained enterocytes was patchy and in discrete areas in the villi of the duodenum and jejunum (Figures 2.3 A, C, D, E and G) and only a few stained cells were found in the ileum (data not shown). Similar results were obtained with tissues in paraffin sections (Figures 2.3 H and I). No cells were stained in the intestinal tissue samples from the mock-inoculated pig (Figure 2.3 B). In the infected regions, individual epithelial cells or a few clustered cells exhibited viral antigen in their cytoplasm in a punctate pattern (Figures 2.3 C and F). Positive enterocytes were located predominantly at various sites, at the tips (Figure 2.3 D, E, G) or sides of each villus (Figure 2.3 A and C). Viral capsid antigen was detected at different locations within the cell cytoplasm in both whole mount tissues and paraffin sections: in some cases the signal was perinuclear (Figures 2.3 D, E and I) apical (Figure 2.3 E) or diffuse (Figures 2.3 C, D, F and H) but was not observed in the nucleus (in Figure 2.3 D, the signal was perinuclear as confirmed by sectional images, not shown). Positive IF for the viral capsid antigen in paraffin sections was observed in deep
areas of the duodenum in only a few pigs (data not shown). It is unclear whether the antigen was located in Brunner glands or crypts. More pigs need to be analyzed to confirm if replication is occurring there or, if binding and internalization occurred because the putative NoV receptors (HBGA) are expressed in this location.

Small intestinal tissue samples of pigs positive by RT-PCR from 12 of 21 (57%) pigs also tested positive by IF in the group inoculated with P0 (Table 2.2), 4 of 6 (66%) pigs from the P1-inoculated group and 2 of 4 (50%) pigs inoculated with P2. We could not detect the virus by IF in the small intestine of some pigs that were positive by RT-PCR although IF permits evaluation of only a few areas and cells of the small intestine of each pig.

To evaluate if the HuNoV replicated in the small intestinal cells, we used an antibody to the non-structural RdRp viral protein. We detected positive signals in the HuNoV-inoculated pigs (Figure 2.4 B, D) although the distribution of infected small intestinal cells detected by this antibody was limited (data not shown). Fluorescent co-immunolocalization of whole mount intestinal tissues also demonstrated that the RdRp antigen was detected in enterocytes that also exhibited the presence of the capsid protein antigen (Figures 2.4 A, B, D). In some cells that showed positive signal for the capsid antigen, the RdRP antigen was not detected (see asterisks in Figures 2.4 A and D). In addition, although the capsid antigen was detected at various locations throughout the cell cytoplasm, the RdRp antigen localization was only apical and in some cases formed globular-like structures (see arrows in Figures 2.4 B and D).
2.4.3 Histopathologic examination

Intestinal segments of HuNoV (n=7) and mock-inoculated (n=2) pigs euthanized from PID 2-5 were examined for the presence of macroscopic and microscopic lesions. Only 1 of 7 HuNoV P0-inoculated pigs euthanized at PID 4 had mild pathologic changes. These included moderate multifocal villus atrophy (30-40% of the villi), villus enterocytes with low columnar morphology and mild foamy cytoplasm and subtle edema of the lamina propria in the duodenum (not shown). No pathologic changes were seen in the jejunum and ileum.

2.4.4 Apoptosis in the small intestinal tissues of HuNoV-inoculated pigs

Because few infected pigs showed macro and micro small intestinal lesions, we used the TUNEL reaction to evaluate whether infected enterocytes were dying by apoptosis instead of lysis, and if apoptosis was occurring at a higher rate in infected pigs than in controls. Apoptotic cells were counted at a 20 X magnification in 10 villi from a single section for each pig. All of the HuNoV-inoculated pigs showed increased numbers of apoptotic cells per microscopic field (20-70 cells) when compared to mock-inoculated control pigs (3-7 cells) (Figures 2.5 A and B). No pigs treated with dexamethasone were analyzed.

2.4.5 Detection of calicivirus-like particles by TEM in small intestinal tissues

The ultrastructural morphology and the presence of HuNoV-like particles in the small intestine from HuNoV-inoculated (n=10) and mock-inoculated (n=3) Gn pigs was analyzed using TEM (Figures 2.6 A-E). Whereas most of the small intestinal tissues from
HuNoV-inoculated Gn pigs did not show abnormalities, we noted enterocytes with cytoplasmic vesicles of 400nm to 1.5µm in diameter (see arrows on Figure 2.6 A, Ve) containing calicivirus-like particles of 25-40 nm in diameter (Figures 3 C and D) in the small intestinal TEM section of 3 pigs at PID 3 (2 pigs from group A and 1 from group C). These particles were seen within or next to membrane structures in the cytoplasm (Figures 2.6 C, D and E). No viral particles were found in the nucleus. Some cells also had changes in their intracellular organization with nuclear displacement and decrease of organelle numbers (Figures 2.6 A and C). Such vesicles and calicivirus-like particles were not present in tissue samples from the mock inoculated pigs (Figure 2.6 B).

2.5 DISCUSSION

Mild diarrhea was observed in most but not all HuNoV-inoculated pigs from the same litter, suggesting that genetic variability among individuals may play a role in pigs as it does in humans (37). The incubation period was 24-48 hrs, the diarrhea was mild and of short duration (1 to 3 days) similar to what has been described in human volunteers (12, 52). Thus, the HuNoV in Gn pigs caused an acute and self-limiting disease as it does in its original host. Detection of fecal virus shedding was short (1-4 days by RT-PCR, but longer by amplicon hybridization, through PID 8). Longer asymptomatic shedding has been described in some normal humans, where 28% of infected individuals shed virus for up to 3 weeks after onset of disease (36). Similar results were observed in a trial involving pigtail macaques inoculated with Toronto virus (HuNoV GII/3) (43). The possibility that we were detecting the pass-through inoculum in the small intestines can not be excluded for day 1, but detection beyond this time is
improbable. In addition, we couldn’t detect viral antigen, viral RNA or antibodies to HuNoV in 2 pigs inoculated orally with inactivated virus (data not shown). This is in contrast to what was described in a study where common marmosets and tamarins orally inoculated with Norwalk virus shed virus for 3 to 4 days but no clinical signs or antibody responses were detected so it remained unclear whether viral replication occurred (37). In our study, most of the pigs had diarrhea and half of them seroconverted. Moreover, viral antigen was also observed in the cytoplasm of the intestinal epithelial cells of these pigs. In addition, we demonstrated that the viral RdRp was present in the infected cells (Figure 2.3 B), showing that the virus was taken up by the enterocytes and translation of viral nonstructural proteins also occurred. We attempted to mimic an immunosuppressed state with dexamethasone treatment such as that occurring in transplanted individuals. We did not observe an increase in severity or duration of diarrhea or fecal viral shedding with the given dose during the given time frame; therefore, these animals were analyzed with the untreated pigs.

Viral RNA (5/8 pigs) and viral antigen (4/8 pigs) were detected in serum of P0 orally-inoculated pigs on PID 1 or 2, demonstrating the existence of transient viremia. Because no lesions were observed in any organs other than the small intestine, the impact of the viremia on the pathogenesis of the disease is unknown. The pigs that had virus in their serum still developed low serum IgG antibody titers to HS66, maybe because the viremia was very short-lived (less than 1 day).
Similar percentages of viremia were detected in P0, P1 and P2-inoculated pigs in a more extensive study (Souza, Cheetham and Saif, unpublished). Viremia has been detected for other enteric viruses such as the porcine sapoviruses (PEC/Cowden) in pigs, and human rotavirus in humans, mice and pigs without causing lesions outside the small intestines (4, 5, 14).

As described for the PEC/Cowden (14), we report that one pig inoculated intravenously with P0 resulted in viral shedding and diarrhea. However, a P1 intravenously-inoculated pig did not develop infection (data not shown).

In our study seroconversion occurred in 59% of the inoculated pigs with low to moderate antibody titers ranging from 20-200, independent of the inoculum passage used. This was perhaps because the duration and severity of the infection was too limited to induce a strong IgG immune response or due to the insensitivity of our ELISA. Because the antibody titers of Gn pigs were low, we used the baculovirus expressed capsid protein immunostaining assay to further confirm seroconversion. Also, we are reporting IgG seroconversion although some pigs had more prominent serum IgA antibody responses, whereas others had more prominent serum IgG antibody responses (Souza, Cheetham and Saif, unpublished). Because the Gn pigs are completely naïve to these viral antigens, their immune responses cannot be accurately compared to those of adult humans who have likely been repeatedly exposed to NoV since early childhood (31). The antibody responses in humans may be of higher magnitude because, although long term memory may not be as efficient in NoV infections as for other diseases, and complete protection does not exist between strains from different genogroups (23), there are common epitopes within and between genogroups (32) that likely cause a stronger immune response after
repeated NoV exposure. Nevertheless we have also detected specific immune responses from pigs exposed to this GII/4 strain by B cell ELISPOT and cytokine ELISA (Souza, Cheetham and Saif, unpublished, will be presented at The American Society for Virology 25th annual meeting July 15-19, 2006 University of Wisconsin-Madison). Similar findings have been reported for macaques inoculated with Toronto virus (43) and Rhesus monkeys inoculated with Norwalk virus where infection occurred in both the presence and absence of seroconversion (6). In another study, although 3 Rhesus monkeys inoculated with Norwalk virus shed virus in feces, only the one that shed for 19 days seroconverted to Norwalk virus. Therefore, long-term shedding, a high amount of virus replication or repeated exposures to HuNoV may be necessary to induce a strong immune response in naïve animals.

In this study, ELISA, RT-PCR and amplicon hybridization results varied from animal to animal, this is similar to what has been previously described by others when inoculating monkeys with NoV GII/3 Toronto strain (43). Therefore, all 3 tests were necessary to ensure viral detection. We observed both pigs that shed virus without showing signs of diarrhea, and also pigs with diarrhea that were negative for viral shedding. The latter observation maybe due to the viral shedding, being below the detection limit or to the presence of fecal inhibitors. In some cases the effect of RT-PCR inhibitors was diminished by dilution of the RNA, but as a consequence the assay sensitivity was also decreased (47). By IEM, only a few viral particles were detected in only 1 of 15 pigs’ intestinal contents tested at PID 3 indicative of very low concentrations of virus shedding (<10^6 viral particles/ml). Shedding of low quantities of virus has also been reported for humans and monkeys (43).
In some pigs that were positive by RT-PCR, viral antigen was not detected by IF or by TEM. This could be due to the limited intestinal areas inspected for each pig using these techniques, a lower extent of the infection or to the rapid extrusion of infected epithelial cells prior to euthanasia.

Histopathologic examination showed mild lesions in the upper intestine of only 1/7 pigs. We speculate that because of the mild diarrhea and detection of scattered, patchy infected cells by IF, the histopathologic lesions in pigs are subtle or absent. Moreover if most infected cells or bystander cells die by apoptosis, cytopathic lesions in the intestinal tract may be absent. It is likely that in our experiments, the HuNoV HS66 strain did not replicate as efficiently in pigs as in the host species. We expect that adaptation by further serial passage in the pig may increase the pathogenicity for the new host.

In addition, extensive histopathologic lesions may not be apparent in the Gn pig’s intestine because the HuNoV infected enterocytes may be dying by apoptosis and not lysis. All the intestinal tissues from the HuNoV-inoculated pigs examined showed increased numbers of apoptotic cells by the TUNEL assay, when compared to the control pig tissues. Apoptosis has also been described for other members of the Caliciviridae family. Apoptosis of hepatocytes was observed in naturally infected rabbits with RHDV causing severe pathology that usually results in death (3). Apoptosis has also been described for FCV infection of cell cultures (2, 35, 42). Of interest, induction of apoptosis by FCV requires active viral replication (42). Clinical cases of allograft lesions from pediatric intestinal transplant patients were common to allograft rejection, but increased superficial apoptosis was characteristic of HuNoV enteritis, whereas crypt apoptosis was
common to both. Apoptotic bodies were present in epithelial cells and macrophages containing apoptotic bodies were observed in the villus lamina propria. Surprisingly, in the Gn pigs examined, the extrusion zones of the villi of both infected and uninfected pigs did not show many apoptotic cells. This may be because the villus enterocyte turnover rates in Gn pigs are slower than in conventional pigs of similar ages (29). Moreover, in mouse small intestine, shedding of enterocytes from these zones usually occurs prior to detectable cellular activation of caspase 3 or nuclear condensation (50). Data from a microarray study of mouse gene expression in intestinal epithelial cells along the crypt-villus axis supports these findings (24). No up-regulation of apoptosis related genes was observed in the villous enterocytes although more than a thousand genes were differentially expressed.

We observed different patterns of viral antigen distribution within the cell (perinuclear, apical or diffuse) that may represent different stages of the viral replication cycle. The RdRp antigen was detected in the apical portion of the enterocytes, forming in some cases globular-like structures (Figure 2.4 B). We speculate that these may be the membranous vesicles containing calicivirus-like particles that were observed by TEM (Figures 2.6 A and C). As described for other positive-stranded RNA viruses that replicate in association with cellular membranes (39), these globular structures or vesicles may represent virus replication sites. The membranous vesicles containing the calicivirus-like particles may have originated from a cellular organelle as described for other members of the Caliciviridae family such as Feline Calicivirus (FCV) (13). In in vitro experiments, a non-structural protein encoded by the N-terminal region of ORF1 of both GI (Norwalk virus) and GII (MD145 GII/4) NoVs was associated with Golgi
apparatus localization and there was a discrete time course in the disassembly of the Golgi complex into aggregates generating 2 patterns: one of marked discrete aggregates and the other a more diffuse distribution of the protein throughout the cytoplasm (10).

The TEM images showed cells with morphologic changes and the presence of viral particles similar to what has been described for Murine NoV 1 (MNV-1) infection of macrophages in vitro (51). Contrary to what has been described for infection of epithelial cells in cats by FCV (33), which belongs to the Vesiviruses genus, no viral particles were observed in the nucleus.

In humans, genetic factors such as polymorphisms in the FUT1 (H), FUT2 (Se) and FUT3 (Le) genes that code for glycotransferases responsible for producing carbohydrate chains have been associated with susceptibility to NoV GI and II (22, 25, 26). At present we are evaluating reagents and assays to test for HBGA in pigs to ascertain if the same or similar genetic factors influence NoV infection of Gn pigs.

Similar to the human population, pigs infected with HuNoVs showed various degrees of resistance, susceptibility and presence or absence of seroconversion, severity and duration of clinical signs and viral shedding. For these reasons the Gn pig model may be useful to study the pathogenesis of HuNoV infections. Because less than 100% of the pigs became infected, further serial pig passage of virus and other variables may need to be assessed to increase infection and diarrhea rates in this model (pig HBGA types, etc). Nevertheless, serial passage of the virus in pigs (3 passages), with virus shed at each passage, and detection of HuNoV-infected cells by IF suggests that the HuNoV from GII/4 is at least partially adapting to replication in the Gn pig host and that further adaptation may increase its pathogenicity for the pig.
2.6 ACKNOWLEDGMENTS

Thanks to Menira Souza, Drs. T. Meulia and S. Grimes for their help with this work; Trang Van Nguyen, Marli Azevedo for their suggestions on statistical analysis and Veronica Costantini for technical assistance. We also thank Dr. J. Hughes, who kindly provided the original NoV GII/4 human fecal sample; Dr. M. Estes, who kindly provided the NS14 MAb; Dr. K. Green, who kindly provided MD145 VLPs and antiserum to MD145 VLPs and antiserum to MD145 RdRp and Dr. J. Xiang for confirming some preliminary RT-PCR results. Dr. J. Hanson and R. McCormick provided animal care. We thank Sean Smith, Scott Fox and Dr. Jeff Hayes at the Animal Disease Diagnostic Laboratory, Ohio Department of Agriculture, Reynoldsburg, OH. for providing assistance with the IHC technique. We also thank Andrea Kaszas and David Fulton at the Molecular and Cellular Imaging Center who provided technical assistance with the confocal and electron microscope and Dr. Jackwood and Dr. Theil for reviewing the manuscript. Salaries and research support were provided by state and federal funds appropriated to the Ohio Agricultural Research and Development Center, The Ohio State University. This work was funded by National Institute of Allergies and Infectious Diseases, National Institute of Health, Grant # AI49742.
2.7 REFERENCES


<table>
<thead>
<tr>
<th>Group (Inocula)</th>
<th>Genus</th>
<th>Genogroup (G)</th>
<th># of pigs</th>
<th>Route of inoculation</th>
<th>Diarrhea Onset (PID)</th>
<th>Duration (days)</th>
<th>Fecal Virus Shedding Onset (PID)</th>
<th>Duration (days)</th>
<th>Seroconversion Ab^b ELISA</th>
</tr>
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<tr>
<td>Hou90</td>
<td>SaV</td>
<td>I</td>
<td>3</td>
<td>2 PO, IVd</td>
<td>3/3</td>
<td>1-2</td>
<td>5-10</td>
<td>2-3</td>
<td>2-3</td>
</tr>
<tr>
<td>NV</td>
<td>NoV</td>
<td>I</td>
<td>1</td>
<td>IV</td>
<td>1/1</td>
<td>2</td>
<td>1</td>
<td>0/1</td>
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<td>NoV</td>
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<td>3</td>
<td>2 PO, IV</td>
<td>2/3</td>
<td>4</td>
<td>1-2</td>
<td>2/3</td>
<td>3-4</td>
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<tr>
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<td>NoV</td>
<td>II</td>
<td>4</td>
<td>3 PO, IV</td>
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<td>3</td>
<td>2 PO, IV</td>
<td>3/3</td>
<td>3-4</td>
<td>1-2</td>
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Table 2.1 Pilot study with different human enteric caliciviruses (Norovirus and Sapovirus) strains in gnotobiotic pigs

- Post inoculation day
- Four-fold increase in NoV GII/4-specific IgG antibody measured by antibody ELISA
- Per oral
- Intravenous
- Norwalk virus
<table>
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<tr>
<th>Group</th>
<th>Inoculum&lt;sup&gt;a&lt;/sup&gt;</th>
<th>No. of pigs</th>
<th>Euthanasia (PID&lt;sup&gt;b&lt;/sup&gt;)</th>
<th>Diarrhea at euthanasia (%)</th>
<th>IC&lt;sup&gt;c&lt;/sup&gt; (+) RT-PCR (%)</th>
<th>IC&lt;sup&gt;c&lt;/sup&gt; (+) Ag&lt;sup&gt;d&lt;/sup&gt; ELISA (%)</th>
<th>IF&lt;sup&gt;e&lt;/sup&gt; gut tissues (%)</th>
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<tbody>
<tr>
<td>A</td>
<td>Passage 0</td>
<td>16</td>
<td>2-3</td>
<td>12/16 (75)</td>
<td>9/16 (56)</td>
<td>6/16 (38)</td>
<td>8/14 (57)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>4-5</td>
<td>8/10 (80)</td>
<td>2/10 (20)</td>
<td>2/10 (20)</td>
<td>4/7 (57)</td>
</tr>
<tr>
<td>B</td>
<td>Passage 1</td>
<td>5</td>
<td>2-3</td>
<td>4/5 (80)</td>
<td>2/5 (40)</td>
<td>1/5 (20)</td>
<td>2/3 (67)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>4-5</td>
<td>5/6 (83)</td>
<td>1/6 (17)</td>
<td>0/6 (0)</td>
<td>2/3 (67)</td>
</tr>
<tr>
<td>C</td>
<td>Passage 2</td>
<td>6</td>
<td>2-3</td>
<td>3/6 (50)</td>
<td>3/6 (50)</td>
<td>0/6 (0)</td>
<td>2/4 (50)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>32/43 (74)</td>
<td>17/43 (40)</td>
<td>9/43 (21)</td>
<td>18/31 (58)</td>
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<td>Mock</td>
<td>8</td>
<td>2-5</td>
<td>0/8</td>
<td>0/8</td>
<td>0/8</td>
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Table 2.2. Detection of HuNoV GII/4 HS66 virus by RT-PCR and antigen ELISA of intestinal contents (IC) and indirect immunofluorescence of small intestinal tissues of HuNoV-inoculated and mock-inoculated Gn pigs euthanized in the acute phase of disease (PID 2-3 and PID 4-5).

<sup>a</sup> Oral inoculation using 5 ml 1:10 stool filtrate or serial passaged intestinal contents (IC). Passage 0: original sample; P1: IC RT-PCR positive from a pig inoculated with P0; P2: IC RT-PCR positive from a pig inoculated with P1; number of positive pigs/ total number of pigs tested.

<sup>b</sup> Post-inoculation day (PID).

<sup>c</sup> Intestinal contents (IC).

<sup>d</sup> Antigen ELISA (Ag), number of positive pigs/ total number of pigs tested.

<sup>e</sup> Indirect immunofluorescence (IF), number of positive pigs/ total number of pigs tested.

<sup>f</sup> Mock inoculation with 5 ml EMEM.
<table>
<thead>
<tr>
<th>Group</th>
<th>Inoculum&lt;sup&gt;a&lt;/sup&gt;</th>
<th>No. of pigs orally inoculated</th>
<th>Diarrhea (%)</th>
<th>Mean onset of diarrhea (PID&lt;sup&gt;b&lt;/sup&gt;)</th>
<th>Mean duration of diarrhea (days)</th>
<th>Fecal virus shedding (%)</th>
<th>Mean onset of fecal virus shedding (PID&lt;sup&gt;b&lt;/sup&gt;)</th>
<th>Mean duration of fecal virus shedding (days)</th>
<th>Seroconversion&lt;sup&gt;c&lt;/sup&gt; (%)</th>
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<tbody>
<tr>
<td>A</td>
<td>Passage 0</td>
<td>10 (+1 I.V.&lt;sup&gt;d&lt;/sup&gt;)</td>
<td>7/11 (64)</td>
<td>2</td>
<td>2</td>
<td>8/11 (73)</td>
<td>1</td>
<td>3</td>
<td>7/11 (64)</td>
</tr>
<tr>
<td>B</td>
<td>Passage 1</td>
<td>6 (+1 I.V.&lt;sup&gt;d&lt;/sup&gt;)</td>
<td>6/7 (86)</td>
<td>2</td>
<td>3</td>
<td>2/7 (29)</td>
<td>1</td>
<td>2</td>
<td>4/7 (57)</td>
</tr>
<tr>
<td>C</td>
<td>Passage 2</td>
<td>4</td>
<td>3/4 (75)</td>
<td>2</td>
<td>1</td>
<td>2/4 (50)</td>
<td>2</td>
<td>2</td>
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<tr>
<td>Total</td>
<td></td>
<td>22</td>
<td>16/22 (73)</td>
<td>2</td>
<td>2</td>
<td>12/22 (55)</td>
<td>1.3</td>
<td>2.3</td>
<td>13/22 (59)</td>
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<td>0/6</td>
<td>N/A&lt;sup&gt;f&lt;/sup&gt;</td>
<td>N/A</td>
<td>0/6</td>
<td>N/A</td>
<td>N/A</td>
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Table 2.3. Diarrhea and fecal virus shedding detected by RT-PCR, and seroconversion detected by antibody ELISA in Gn pigs inoculated with HuNoV GII/4 HS66 strain or mock-inoculated Gn pigs maintained through PID 21.

<sup>a</sup> Oral inoculation using 5 ml 1:10 human stool filtrate (HS66) or serial passaged intestinal contents (IC). Passage 0: original sample; P1: IC RT-PCR positive from a pig inoculated with P0; P2: IC RT-PCR positive from a pig inoculated with P1

<sup>b</sup> Post-inoculation day (PID).

<sup>c</sup> Detected by antibody ELISA (IgG) (Ab), number of positive pigs/ total number of pigs tested.

<sup>d</sup> One pig was inoculated by intravenous (I.V.) route.

<sup>e</sup> Mock inoculation with 5 ml EMEM.

<sup>f</sup> Not applicable.
Figure 2.1 Immune electron micrograph of HS66 VLPs incubated with guinea pig MD145 VLPs polyclonal antiserum (1:500). Bars 100 nm and 50 nm.

Figure 2.2 Western blot of CsCl-gradient purified HS66 (GII/4) VLPs. Lane 1: molecular weight marker (kDa), lane 2: HS66 VLPs, lane 3: negative control (40% sucrose cushion purified Sf9 insect cell proteins). The double band of 58-60 kDa has been described for other NoV VLPs from GI and II may be result of a translation product from an internal initiation codon in ORF2. The bands of 20-25 kDa probably are result of cleaved product also described by Kitamoto et al. (reference # 223).
Figure 2.3 Confocal microscopy showing indirect immunofluorescent localization of the HuNoV capsid protein in small intestinal tissues from virus-inoculated Gn pigs.
Figure 2.3 legend. A-G) IF on whole mount tissues; H-I) IF on tissue paraffin sections. Primary antibody NS14 MAb was used to detect the capsid protein of HuNoV GII. Secondary antibody: goat anti-mouse IgG Alexa488 (green); nuclear counterstain (A-G) Sytox orange (red) or (H-I) propidium iodide (red); and actin stained with phallotoxin Alexa633 (blue). A) Jejunum of a P2-inoculated Gn pig at PID 3 showing scattered IF positive cells on the villi tips or sides. B) Jejunum of a mock-inoculated pig with no IF positive cells evident. C) Jejunum of a P1-inoculated Gn pig at PID 3, showing individually infected enterocytes on the side of a villus. D) Tip of a villus in the duodenum of a P1-inoculated Gn pig at PID 3 with several contiguous infected cells. E) Tip of a villus on Jejunum of a P1-inoculated Gn pig at PID 3 with positive signal in the apical portion of the enterocyte cytoplasm. F) Enterocytes from duodenum of a P0 inoculated Gn pig at PID 3, showing nuclear displacement and positive signal throughout the cytoplasm of individual cells. G) Tip of a villus in duodenum of a P0-inoculated pig at PID 2 showing contiguous infected cells. H) Paraffin section of duodenum of a P0 inoculated Gn pig at PID 4 show contiguous infected enterocytes on the sides of the villi and some individual enterocytes on the tips. I) Higher magnification, most enterocytes showing perinuclear IF. Bars: A,B=100 µm; C-H =20 µm.
Figure 2.4 Confocal microscopy of immunofluorescent colocalization of viral capsid and RdRp antigens in whole mount tissue. A) Anti-capsid NS14 MAb with the secondary goat anti-mouse IgG antibody conjugated to Alexa488 (green) and B) Anti-viral RdRp guinea pig serum with the secondary goat anti-guinea pig IgG labeled with AlexaFluor576/603 (red) C) Cell nuclei were counterstained with TOPRO-3 iodide (642/661) dye (blue) D) Merged image where the yellow color indicates colocalization. Asterisks indicate cells where only the capsid protein was detected and arrows indicate apical globular-like structures detected with the anti-RdRp serum Bars =20 µm.

Figure 2.5 A) Immunohistochemistry (IHC) using TUNEL reaction, more cells with apoptotic nuclei (arrows) were observed in an HuNoV-inoculated Gn pig duodenum section in a similar pattern as the virus infected cells from Figure 1 E. B) IHC TUNEL results showing apoptotic cells in a mock-inoculated Gn pig control. Bars=50 µm.
Figure 2.6 Transmission electron microscopy of A) jejunum of a HuNoV GII/4 (Passage 1) inoculated Gn pig at PID 3. The arrows indicate vesicles not seen in the control Gn pig. The nucleus is displaced apically and cells have changes in their intracellular organization. The cellular organelles are decreased in number. B) Jejunum of an age-matched mock-inoculated control. C-E Duodenum of a Passage 1-inoculated Gn pig at PID 3 C) The overall intracellular morphology of the enterocyte is disrupted, with vesicles (Ve) in the cytoplasm containing calicivirus-like particles (arrow) and nuclear (Nu) displacement. A Goblet cell can be observed on the side D) Detail of membrane vesicle from figure C containing calicivirus-like particles. E) Aggregates of calicivirus-like particles in the cytoplasm of another cell. Bars: A-C=1 μm. D=200nm E=225nm.
CHAPTER 3

BINDING PATTERNS OF HUMAN NOROVIRUS-LIKE PARTICLES TO BUCCAL AND INTESTINAL TISSUES OF GNOTOBIOTIC PIGS IN RELATION TO A/H HISTO-BLOOD GROUP ANTIGEN EXPRESSION

3.1 SUMMARY

Histo-blood group antigen (HBGA) phenotypes have been associated with susceptibility to human Noroviruses (HuNoV). Our aims were to determine A/H HBGA expression in buccal and intestinal tissues of gnotobiotic (Gn) pigs; to determine if virus-like particles (VLPs) of HuNoV genogroup I and II bind to A or H type tissues; and to compare A/H expression and VLP binding patterns and confirm their binding specificities by blocking assays. A hemagglutination inhibition test using buccal cells from live pigs was developed to determine the Gn pig’s A/H phenotype to match viral strains with previously determined HuNoV VLP binding specificities. We also determined the A/H phenotypes and compared this data to the infection outcome of 65 Gn pigs inoculated with HuNoV GII/4 HS66. The pigs expressed A and/or H or neither
antigen on their buccal and intestinal tissues. The HuNoV GI/GII VLPs of different clusters bound to tissues from 4 pigs tested (2 A+; 2 H+). The GI/1 and GII/4 VLPs bound extensively to duodenal and buccal tissues from either A+ or H+ pigs, but surprisingly GII/1 and GII/3 VLPs bound minimally to duodenum of an A+ pig and GI/3 bound to the duct lining of an H+ pig. The VLP binding was partially inhibited by A, H1 or H2 specific monoclonal antibodies, but was completely blocked by porcine mucin. Of the 65 HS66-inoculated Gn pigs, although not significantly different, more A+ and H+ pigs shed virus (47%) compared to the non A+/non H+ pigs (25%). Based on seroconversion in 22 convalescent pigs, significantly more A+ or H+ pigs (79%) than the non-A+/H+ pigs (25%) seroconverted.

3.2 INTRODUCTION

Recently, differential susceptibility of humans to Norovirus (NoV) infection has been reported, depending on their histo-blood group antigen phenotypes (HBGA) (16). The HBGA are terminal disaccharides added in a stepwise manner to precursor carbohydrate chains by the action of different glycosyltransferases (29). Inactivating mutations in the glycosyltransferase gene at the ABO(H) locus results in the O phenotype that represents the H precursor without any further carbohydrate addition; thus, presence of the H antigen with absence of A or B antigens corresponds to the O phenotype. The addition of different terminal disaccharides to the H chain results in either the A or B antigens. Although these antigens were first described on the surface of human red blood cells (RBC), their expression occurs throughout the body. The FUT2 gene codes for a
glycosyltransferase that determines the secretor (Se) phenotype of an individual, and when active, this enzyme mediates the expression of the ABO(H) antigens on mucosal epithelial cells and their secretion into body fluids (29). Activity of the FUT2 gene has been linked to the differential susceptibility of individuals to Norwalk virus, a GI NoV; consequently, the Se+ phenotype determines Norwalk virus susceptibility (20). About 20% of individuals have FUT2 inactivating mutations, resulting in a non-secretor (Se−) phenotype which results in resistance to Norwalk virus infection (23, 38). These results coincide with the finding that volunteers with an active Se+ gene were 40 times more likely to become Norwalk virus infected than Se− individuals (20).

A hemaglutination assay (HA) using human and chimpanzee RBC established that Norwalk virus VLPs differentially agglutinate RBC of different blood groups, agglutinating all A, O and AB samples but only 4 of 14 type B samples (17). These results correspond to the findings obtained from a Norwalk virus challenge study in volunteers (15). Also, Norwalk virus VLPs bind to HBGA present on gastroduodenal epithelial cells of Se+ but not Se− individuals (23). In vitro studies have also shown different HBGA binding patterns for other HuNoV VLPs from both GI and GII (11).

Expression of HBGA on RBC is a recent evolutionary event, being present only in humans and anthropoid apes (28). However these antigens are present on enterocytes of other mammals (1). Pigs express A and H antigens on their tissues (32), and also have a gene homolog to the human FUT2 gene that determines the Se status, the guanosine diphosphate (GDP)-L-fucose:β-D-galactoside α-2-L-fucosyltransferase that was identified in porcine submaxillary gland (36). Swine also express A, H or I antigens in
their gut epithelial brush border (1). The I antigen lacks the terminal fucose residue that characterizes the H antigens and therefore fails to react with monoclonal antibodies (MAbs) to A or H antigens. Therefore, VLPs from various HuNoVs strains might bind to swine tissues expressing A or H antigens and if so, this binding should be blocked with A or H-specific MAbs or mucins containing these carbohydrates which may aid in confirming their binding specificities.

In humans histo-blood group typing is readily performed using human RBC. This method is not reliable for pigs as the A/H antigen levels present on swine RBC is low (40). Thus, a more reliable test is needed to determine the pig’s A/H phenotype for HuNoV studies prior to inoculation, to match the porcine A/H phenotype with comparable phenotype-specific HuNoV strains and to evaluate the role of these antigens in differential susceptibility of swine to HuNoV strains.

Previously, in our HuNoV pathogenesis study, we observed that 44% of the 65 Gn pigs orally inoculated with HS66 shed virus in their feces and 59% seroconverted to the HuNoV strain (S. Cheetham, M. Souza, T. Meulia, S. Grimes, M.G. Han and L.J. Saif, Abstr. 24th American Society for Virology, abstr W50-8, 2005 and submitted for publication). The HS66 strain used in that study belongs to GII/4, the most common cluster circulating in humans worldwide. In this study we examined the A/H phenotype of the 65 HS66-inoculated pigs by indirect immunofluorescence (IF) or immunohistochemistry (IHC) and compared this data to the infection results to determine the relationship between A/H phenotype and susceptibility to HS66 infection. We observed that pigs that expressed either A or H antigen on the intestinal mucosa had
significantly higher rates of diarrhea and seroconversion to the NoV strain, and fecal virus shedding was also higher although not significantly. Therefore, in pigs as well as humans the HuNoV VLP binding may be indicative of susceptibility to NoV infection.

3.3 MATERIALS AND METHODS

3.3.1 Animals

A total of 79 Gn pigs (5-50 days-old), including the 65 virus-inoculated and 14 mock-inoculated controls were delivered and maintained as previously described (25). The Gn pigs were of Hampshire breed and they were used to study the pathogenesis of a HuNoV GII/4 in the Gn pig model (S. Cheetham, M. Souza, T. Meulia, S. Grimes, M.G. Han and L.J. Saif, Abstr. 24th American Society for Virology, abstr W50-8, 2005 and submitted for publication). In the present study, the 79 Gn pigs’ archival paraffin embedded duodenal and buccal tissues were typed for their A and/or H antigen expression. Ten additional age-matched conventional pigs from a different herd (cross of Berkshire and Landrace cross-breeds) were also tested for their A/H phenotype to address whether A/H phenotype frequency distribution was dependent on the herd and breed of origin. Tissues from 4 of the 14 mock-inoculated Gn pigs were also used in the VLP binding and blocking assays. A subset of the Gn pigs (n=38), as well as 2 sows (from the Hampshire herd), were tested for their expression of HBGA on buccal epithelial cells from the live pigs.
A/H phenotyping by immunofluorescence (IF) and immunohistochemistry (IHC) of intestinal and buccal tissues in paraffin sections

Archival paraffin blocks containing samples of intestinal and buccal tissues were sectioned. Slides were kept at 60°C for 20 minutes, deparaffinized in xylene twice for 5 minutes and rehydrated through the graded ethanol series. To unmask the antigens, proteinase K (DAKO, Carpinteria, CA) treatment was applied for 3 minutes, then the slides were washed in PBS buffer (10mM potassium phosphate, 150 mM NaCl, pH7.4) and blocked with 1% normal goat serum for 20 minutes at room temperature (Rt). Then the slides were incubated with either MAb to human blood group A (undiluted) derived from a single clone line Birma-I (Immucor, Norcross, GA), BG-4 MAb to human H1 (1:100) (Signet, MA) or BRIC 231 MAb (1:100) (Biogenesis, U.K.) which recognizes the human H2 antigen, for detection and subtyping of A, H1 and H2 antigens. A goat anti mouse IgG conjugated to Alexa488 (green) at a 1:500 dilution (Invitrogen, Carlsbad, CA) was used as secondary antibody for 1 hr at Rt. Cell nuclei were counterstained with propidium iodide (red) at a concentration of 3 µg/ml and the slides were then observed using the Leica TCS-SP (Leica, Wetzlar, Germany) laser scanning confocal microscope.
In a variation of this protocol used for IHC, blocking for endogenous peroxidase was performed with 0.3% H$_2$O$_2$ in methanol for 10 minutes at Rt and the secondary antibody used was a goat anti-mouse IgG conjugated to horseradish peroxide (HRP) (1:1000) (DAKO, Carpinteria, CA). The reaction was developed with 3-amino-9-ethylcarbazole (AEC) substrate (Vector Labs, Burlingame, CA), counterstained with Mayer’s hematoxylin and viewed using a light microscope.

3.3.3 NoV VLP binding and blocking assays using tissue slides

The slides were deparaffinized and the antigens unmasked as described above. The slides were incubated with NoV VLPs of Norwalk virus (NV) GI/1 (kindly provided by Dr. M.K. Estes, Baylor College of Medicine, TX), Desert Shield Virus (DSV) GI/3, Hawaii Virus (HV) GII/1, Toronto Virus (TV) GII/3 (kindly provided by Dr. S. Monroe, CDC), MD145 GII/4 (kindly provided by Dr. K. Green, NIH) or HS66 GII/4 (produced in our lab) at a concentration of 5 µg/ml in PBS buffer and incubated at Rt overnight. Detection of the VLPs was performed using rabbit or guinea pig antiserum specific to each VLP (1:500) (also provided by Drs. Estes, Monroe and Green) followed by the goat anti-rabbit or anti-guinea pig IgG Alexa488 (green) 1:400 dilution (Invitrogen) for 1 h at Rt. Cell nuclei were counterstained with propidium iodine. For the blocking assays, prior to the incubation with the NoV VLPs, slides were first incubated with anti A, H1 or H2 specific MAbs as described for the A/H typing assay and then incubated with the above VLPs.
A control experiment included an additional step of preincubation of each VLP type with its specific VLP antiserum using the same conditions (time and temperature) and concentrations as described above before addition to the slides.

For the inhibition experiments using mucins from porcine stomach (type III) and from bovine submaxillary glands (type 1-S) (Sigma, St. Louis, Mo), a concentration of 1µg/ml mucins were incubated with the VLPs for 1hr at 37°C, prior to being added to deparaffinized and rehydrated slides. Prior to use, mucins were first typed for their content of A/H1/H2 carbohydrates by ELISA. Briefly, porcine and bovine mucins (1 µg/ml) were coated onto duplicate wells in coating buffer (0.05M carbonate buffer pH 9.6) and plates were incubated at 4°C overnight. Two percent skim milk in PBS buffer was added for 1 hr at 37°C. After washing, the slides were then incubated with either MAb to type A (1:10) or MAb BG-4 to H1 (1:100) or MAb BRIC 231 to H2 (1:100) primary antibodies for 2 hrs at 37°C. A goat anti-mouse IgG conjugated to HRP (1:2000 dilution) (DAKO) was used as secondary antibody for 1 hr at Rt, and the assay was developed with tetramethylbenzidine (TMB) (KPL, Gaithersburg, MD) following the manufacturer’s instructions. Plates were washed 4 times between each step with PBS containing 0.5 % Tween 20. Positive samples were those with an absorbance equal or greater to the cutoff value which was defined as the mean of the negative control wells (no primary antibody and primary MAb to an unrelated epitope (spike protein of Transmissible Gastroenteritis Virus, TGEV, 25C9) (33) plus 3 times the standard deviation.
3.3.4 Hemagglutination inhibition (HI) test for A/H typing of pig buccal cells

An HI test (Figure 3.1) was used to determine the pig’s A/H phenotype by using buccal cells collected from live pigs. This cells from the keratinized oral squamous epithelium express A and H2 antigens, but not H1. The HI procedure was adapted from a previously described method (12). Briefly, sterile cotton tipped swabs were used to vigorously swab the inside of the mouth and were then placed in a 15 ml tube containing PBS (pH 7.4). After twirling and removing the swab from the tube, tubes were centrifugated at 120 X g for 15 minutes at 4°C. The supernatant was discarded and the pelleted cells were washed twice in PBS. The buccal cell numbers were standardized to 1.5x10^5 cells per well after adjusting the dilution according to the cell count in a Neubauer’s counting chamber and were added to duplicate wells of V-shaped microtiter plates (Nalge, Nunc, Rochester, NY). Next, MAb Bric231 to H2 (1:50) or the MAb to A (1:10) were added to the wells and the plates were incubated for 1hr at 37°C. The human RBC of A or O (H) types (Immucor) were diluted to 0.5% in veronal buffer and incubated for 2 hrs at 4°C with the RBC for type A and overnight at 4°C for the type H RBC. Subsequently, plates were visually read and results were recorded.

In this test, if the buccal cells from a type A^+ pig bind to the A typing MAb then the MAb is no longer available to bind human A^+ RBC so that when A^+ human RBC are added, they form a dot in the V-shaped well of a microtiter plate. In contrast, when buccal cells from a type H^+ pig are incubated with the MAb to type A, this A MAb should not bind to the cells so that when A^+ human RBC are added, the MAb binds the RBC and forms a lattice in the V-shaped wells. The same concept is applied when A^- or
H⁺ buccal cells are incubated with MAb to H2 prior to addition of human type O (H⁺) RBC. The method and interpretation of results are outlined (Figure 3.1). Human RBC as well as keratinized epithelial cells from the oral mucosa have no H1 but high H2 levels on their surface; therefore, the test was standardized using the MAb to H2. Although the MAb to H2 cross-reacts with A1 RBC (as described by the manufacturer), this outcome can be clearly identified: A⁺ pigs are reactive only with MAb to A, whereas H2⁻ pigs may be reactive with MAbs to both A and H2. For this test a dot means positive HI by the pig’s buccal cells and a lattice means negative HI.

3.3.5 Statistical analysis

The Fisher’s exact test was performed to assess the statistical significance of the proportion of A or H phenotype pigs or the A/H-expressing compared to the non-A/H expressing phenotypes that seroconverted, had shedding and/or diarrhea (Table 3.2). Statistical significance was assessed at a P value of <0.05. Statistical Analysis Systems (SAS Institute Inc., Cary, NC) was used to analyze the data.

3.4 RESULTS

3.4.1 A/H antigen distribution in swine intestinal and buccal tissue sections of gnotobiotic pigs by IF and IHC

Monoclonal antibodies to human HBGA (A, H1, H2) recognized the swine homologs. Distribution of the A and H antigens in the intestinal and buccal tissues varied widely among the 79 Gn pigs tested, but general patterns could be identified. As depicted
for IHC and IF results, in A⁺ pigs, the apical surface of some enterocytes, the whole cytoplasm in goblet cells as well as crypt cells and Brunner glands in duodenum reacted strongly with the MAb to type A (Figures 3.2 A, B and 3.3 A). In some pigs the jejunum and ileum were negative, whereas in others they were positive but with fewer reactive cells, goblet cells and enterocytes (Figures 3.3 D and E). The presence of HBGA in sections of the distal small intestine did not relate to the Gn Pigs’ ages examined at 5 to 50 days post-derivation. Most A⁺ pigs also expressed H antigens on some enterocytes or crypt cells (Figures 3.3 B and C) or in their Brunner glands (Figure 3.3 F). In the buccal tissues, the A antigen expression was observed in the spinous cell layer (Figure 3.4 C) and in the minor salivary glands and with less intensity in the duct linings (not shown).

In the H⁺ pigs, enterocyte apical surfaces, some goblet cell cytoplasm, and some crypt cells (Figure 3.3 I) and Brunner glands (not shown) reacted with the H MAbs. The H⁺ pigs did not express any A antigen (Figure 3.3 G), but the H1 and H2 expression varied in their distribution and amount in the duodenum and buccal tissues within each pig with some pigs expressing either H1 or H2 (Figures 3.3 H, I). In the buccal tissues, H2 was strongly expressed in the parabasal cell layer (Figure 3.4 D), the duct linings and more weakly in the minor salivary glands (Figure 3.4 S). The H1 was expressed in some cases in the same location as H2 (duct linings) and in some cases in the same tissue but with a different pattern (spinous layer) and in some cases H1 was not expressed (data not shown).
3.4.2 NoV GI and GII VLPs binding to swine buccal and intestinal tissues

The VLP binding assays and blocking assays were performed on duodenal and buccal tissues from 4 Gn pigs of known phenotype (2 A+ and 2 H+). The NV (GI/1), HV (GII/1), TV (GII/3), HS66 and MD145 (both GII/4) VLPs attached only to the duodenum (Figures 4 A, E, I, M) and not to distal regions of the small intestine of A+ pigs (not shown). The DSV VLPs did not attach to any sections on the A+ pigs (not shown). The HS66 VLPs had a similar regional and tissue distribution as MD145 VLPs (Table 3.1). The VLPs of each genotype showing binding attached with a unique pattern to the same A+ pig tissue in consecutive sections from the same block (Figures 3.4 A, E, I, M). The binding was extensive for NV, HS66, MD145 VLPs (Figures 3.4 A, M) in both A+ pigs, more limited for HV and with only a few cells staining for TV in only one of the A+ pigs (Figures 3.4 E, I) (Table 3.1). Binding to the parakeratinized stratified squamous epithelium of the oral mucosa of an A+ phenotype pig was extensive for NV, MD145 and HS66 VLPs on the portion of the spinous layer where cells are flat (more superficial) (Figures 3.4 G and K).

In the H+ pigs, NV, HS66 and MD145 VLPs bound extensively to H1 and H2 expressing duodenal (Figures 4 B, N) and buccal tissue sections at the spinous–parabasal cell layer junction (Figures 3.4 H, L) (Table 3.1) whereas HV and TV VLPs bound somewhat less extensively to both buccal (photos not shown) and intestinal tissues (Table 3.1 and Figures 3.4 F, H). The DSV attached only to cells in the salivary duct lining in one H+ phenotype pig (Figure 3.4 T) and not to the squamous epithelium (Figure 3.4 P). The binding patterns of NV, HS66 and MD145 VLPs differed between A+ and H+ pigs.
and they were similar to the A and H antigen distribution. For example, in A\(^+\) pigs, NV, MD145 and HS66 VLPs bound to the apical surface of some enterocytes and to goblet cells (Figure 4 I) whereas in H\(^+\) pigs these VLPs attached to the surface of most enterocytes and to lesser extent to goblet cells (Figure 3.4 J). In an H\(^+\) pig that expressed H2 in the Brunner glands, NV and HS66 VLPs attached to these regions whereas there was less signal at such locations in the A\(^+\) pigs that had little expression of H2 in the Brunner glands (not shown). More NV VLPs bound to enterocytes in the pig expressing H1 whereas HS66 and MD145 VLPs bound more to the enterocytes of the pig expressing mainly H2 (Table 3.1).

### 3.4.3 Blocking of the VLP binding to the buccal and intestinal tissues

Incubation of the slides with MAbs to A, H1 or H2 antigens prior to the addition of NV VLPs did not block VLP binding in either of the two H\(^+\) pigs or in one of the two A\(^+\) pigs (Table 3.1, pigs 1, 3, 4). However, the MAbs partially blocked the NV VLPs from binding to the sections of an A\(^+\) pig also expressing H1 and H2 when using the H1 and H2 MAbs for the duodenal tissue and A and H1 MAbs in the buccal tissue (Table 3.1, pig 2). In contrast, the DSV VLPs were inhibited from binding to the buccal tissue duct lining by the H2 MAb whereas the H1 MAb had no effect (Table 3.1, pig 3). The HV VLP binding to an A\(^+\) pig was not affected by prior incubation with A, H1 or H2 MAbs (Table 3.1, pig 1), whereas, there was partial blocking of TV VLP binding by the H1 and H2 MAbs in both the A\(^+\) and H\(^+\) pigs (Table 3.1, pigs 1, 3). For the HS66 and MD145 VLPs, incubation with MAbs to A, H1 or H2 partially prevented binding in the
duodenum of one of the A\textsuperscript{+} pigs (Table 3.1, pig 1) but the results for the other A\textsuperscript{+} pig showed that the H2 MAb only partially blocked the VLP binding (Table 3.1, pig 2). When the A MAb was used in one of the A\textsuperscript{+} pigs, the signal was reduced in the villus epithelium, but not in the crypts (not shown). In the case of the H\textsuperscript{+} pigs, the H1 and H2 MAbs reduced the binding of the HS66 and MD145 GII/4 VLPs to the duodenal sections (Table 3.1 pigs 3, 4). The attachment of HS66 and MD145 VLPs to buccal epithelium and minor salivary glands was not prevented by incubation with type A MAb, but it was reduced by H1 and H2 MAbs prior to VLP addition in the H\textsuperscript{+} pigs (Table 3.1, pigs 3, 4).

When a mixture of MAbs (to A, H1, H2) were used, more extensive, but still incomplete blocking was observed (not shown) suggesting that other factors may be involved in NoV binding to tissues of pigs.

As a control, we also incubated each VLP type with specific antibody to each VLP prior to addition to the sections. After washing and completing the assays, no VLPs were observed binding to any of the tissues confirming the ability of antibodies to each specific VLP to block binding of the VLPs to the intestinal and buccal sections.

The mucins of porcine and bovine origin were typed for HBGA in ELISA (coating with the mucin and detecting with HBGA MAbs). Both bovine and porcine mucin contained high amounts of A antigen whereas only porcine mucin had lower levels of H1 and H2 antigens. The blocking experiment using mucin was performed with NV (GI/1) and MD145 (GII/4) VLPs on duodenum tissue sections of pig 2 A\textsuperscript{+}(H1\textsuperscript{+}H2\textsuperscript{+}) and pig 3 \textbf{H2}\textsuperscript{+} (A\textsuperscript{+}H1\textsuperscript{−}) phenotypes (Table 3.1). Complete blocking was observed for both VLPs in both phenotypes using the porcine mucin whereas bovine mucin did not block
binding of either VLP to either of the HBGA phenotypes (data not shown). It is unknown if factors other than the A/H antigens are responsible for such results. However, from these results, both NV and MD145 VLPs likely bound to the H antigens in either A⁺ or H⁺ pig phenotype rather than to the A antigen in the A⁺ pig and therefore, no blocking was observed with bovine mucin that contained only A antigens.

**3.4.4 Hemagglutination inhibition test to determine pigs’ A/H phenotypes**

The HI test was rapid and reliable to determine the A/H phenotypes using buccal cells of live pigs. Of 40 pigs tested at different ages (6-50 days old and 2 sows), 23 were A⁺ and 8 were H⁺, with 9 unclassified. Most of the unclassified pigs were usually only a few days old. Sampling of these pigs several weeks later in some cases (3 pigs) permitted the phenotype determination. For some pigs whose A/H phenotype was inconclusive by the HI test, their phenotypes were confirmed by IF of their intestinal tissues after euthanasia. As described by others (17) low temperature and neutral pH were essential for agglutination to occur. It is unknown why the H reaction needed a longer time to agglutinate the RBC but it could be related to the density of the antigen (21).

**3.4.5 A/H phenotyping of the pigs from the HuNoV pathogenesis study** (S. Cheetham, M. Souza, T. Meulia, S. Grimes, M.G. Han and L.J. Saif, Abstr. 24th American Society for Virology, abstr W50-8, 2005 and submitted for publication)

To determine the A/H phenotype of the 79 Gn pigs from the HuNoV pathogenesis study (65 HuNoV-inoculated and 14 mock-inoculated control pigs), archival samples
were tested by IF. Forty-eight (60%) of the Gn pigs (n=79) had A⁺ (H⁺/and few cases H⁻) phenotype, 21 (27%) had H⁺ (A⁻) phenotype and the type could not be determined in 10 pigs (13%). Most of the Gn pigs that came from a single closed herd of Hampshire breed were A⁺. When 10 conventional cross-bred Berkshire and Landrace pigs from another herd were tested, 6 (60%) were H⁺, 2 (20%) were A⁻ and 2 (20%) were A and H negative (not shown).

The pigs infected with the HS66 strain as verified by NoV positive IF in intestinal tissues included both type A⁺ and H⁺ pigs (Table 3.2). Seroconversion occurred in 11 of 14 (79%) A⁺/H⁺ pigs, but only 2 of 8 (25%) of the non A⁺/H⁺ pigs from the 22 convalescent pigs tested (Table 2). When A⁺ and H⁺ phenotypes were compared, 10 of 12 and both H⁺ pigs seroconverted, respectively. There was no statistically significant difference in the latter comparison although the number of H⁺ pigs was low (Table 3.2). Virus shedding was detected in 27 of 57 (47%) of the A⁺/H⁺ pigs compared to 2 of 8 (23%) in the non A⁺/H⁺ pig group. Twenty–four of 44 (55%) A⁺ pigs shed virus in their feces compared to 2 of 8 (25%) H⁺ phenotype pigs (Table 3.2). The differences observed between fecal virus shedding rates and HBGA phenotypes were not statistically significant. However, there was a significant difference for diarrhea in the A⁺/H⁺ group (79%) compared to the non A⁺/H⁺ pigs (37.5%). The difference was not significant when analyzing the A⁺ and H⁺ pig phenotypes separately where 35 of 44 A⁺ and 10 of 13 H⁺ pigs had diarrhea.
Therefore, expression of A and/or H antigens in Gn pigs was associated with significantly higher rates of seroconversion to HuNoV and diarrhea. Fecal virus shedding rates were also higher, but not significantly, in the A⁺/H⁺ pigs compared to the non A⁺/H⁺ pigs (Table 3.2).

The expression of A/H on the apical surface was not present in every enterocyte (Figures 2 A and B white arrows). Similarly, as we reported (S. Cheetham, M. Souza, T. Meulia, S. Grimes, M.G. Han and L.J. Saif, Abstr. 24th American Society for Virology, abstr W50-8, 2005 and submitted for publication), the HS66 viral antigen distribution was limited to isolated enterocytes or a few contiguous enterocytes within the duodenum and jejunum. In addition, the presence of viral antigen in deep portions of the intestine, that we observed in only a few pigs (not shown) may coincide with the expression of A/H in the Brunner glands (Figure 3.2 A, black arrows).

3.5 DISCUSSION

In contrast to the 3 major alleles in humans for the ABO(H) locus, pigs have only A and H antigens (40) and these antigens cross-react with MAbs to human A and H types (1) as shown in this study. The histo-group A transferase cDNA isolated from pigs shares 77% nucleotide and 66.7% amino acid identity with the human counterpart (24) and phylogenetic analysis shows that they cluster together (5). The FUT 2 gene (Se) identified in porcine submaxillary gland is closely related to the human FUT 2 gene (36). No inactivating mutations of either FUT 1 (coding for a glycosyltranferase that biosynthesizes the H antigen) or FUT 2 have been described in mammals other than
humans (22). In humans expression of ABO(H) on the villi surface is confined almost exclusively to Se$^+$ (29). According to this information, the majority of the Gn pigs in our pathogenesis study were likely Se$^+$ because they expressed A or H antigens on the surface epithelia of the intestine. Of the fewer pigs that were negative (10 Gn and 2 conventional pigs) a Se$^-$ phenotype was less probable because neither A nor H antigens were seen in the Brunner glands of the intestine that in humans are independent of the FUT2 gene. Most likely, these pigs could have been of the I phenotype (for which we did not test).

Only one pig that was H$^+$ in the buccal glands but negative with both MAbs to A and H types in the intestinal tissues, might have been Se$^-$. This pig that was classified as a non A$^+$/H$^+$ phenotype, did not shed virus and did not seroconvert to HS66 virus. We plan to investigate this case further.

From our observation of A and H antigen distribution in 79 Gn pigs we concluded that in the porcine small intestine, the mucosal surface of the villi which contained enterocytes and goblet cells expressed mainly H type 1 and 2 chains, whereas the crypts of Lieberkuhn and Brunner glands expressed mainly H type 2 chains. In the pig intestinal sections of A$^+$ phenotype, the A antigen was expressed on the surface of epithelial cells as well as in the cytoplasm of goblet cells and also in Brunner glands in most cases. This distribution of HBGA in the pigs was similar to their expression patterns in humans as described previously (29). The variation among animals could be similar to that in humans where the crypts of Lieberkuhn express the ABO(H) antigens without strict correlation with Se status whereas expression in the deep Brunner glands is completely independent of the FUT 2 gene (Se) and depends on the FUT 1 (H) gene activity (29).
In the pig tissues we observed that mucous cells expressed H type 1, 2 and A antigens. In humans, the expression of these antigens is regulated in a Se dependent way. The H type 1 and 2 are localized in mucinogen granules filling most of the cytoplasm whereas type 3 (not tested in our study) can be found in a supranuclear position suggesting localization to the Golgi apparatus. In H⁺ (A⁻) pigs, we did not observe the A antigen whereas in A⁺ pigs we could, in most cases, also detect the H antigen (mainly H2). In humans A⁺ or B⁺ individuals may express A or B antigen in some acini of mucous cells and H antigen in other acini, maybe due to different maturation stages (29).

Norwalk virus and its VLPs bind H and Le antigens (10, 17). This has been determined by in vitro studies (HA, HI and treatment with periodate and synthetic carbohydrates) but also corroborated by human challenge studies (20). Both GI and GII HuNoV have demonstrated differential binding patterns for Lewis, Se and ABO(H) in vitro (13, 14, 35) and also in vivo (11). Binding patterns for VLPs of reference strains that we tested were: NV (GI/1): H type 1, H type 2, H type 3, Lewis d (Le⁣[^d]⁣), Le⁣[^b]⁣ and Le⁣[^y]⁣; MD145 (GII/4): Le⁣[^b]⁣, A/B, H1, H3; HV (GII/1): Le⁣[^b]⁣, TV (GII/3): Le⁣[^b]⁣; DSV (GI/3): not tested (11). Therefore, some strains would be more suitable for experimental animal adaptation, especially those with a broader binding range such as strains from GII/4. In addition, the use of animal species with more similar HBGA to humans such as monkeys and pigs would increase the chance of successful replication.

Lordsdale virus, a strain belonging to GII/4 and reportedly responsible for the majority of the recent outbreaks, binds to H, Le and A and B antigens which may facilitate its transmission to larger numbers of susceptible hosts (11). Strains that cluster
together tend to have similar binding patterns, although no binding predictions can be made within genotypes or between them (14). Therefore, we expected NoV GII/4 HS66 strain, like Lordsdale and MD145 GII/4 strains, to bind to both A/H in humans but we did not know if the pig’s A/H carbohydrates were similar enough in structure to be recognized by the HS66 VLPs or virus. We confirmed the binding of HS66 VLPs to the paraffin-embedded intestinal tissues of both A and H positive pigs and also confirmed infection of pigs of both A and H phenotypes by the HS66 virus. Although the distribution of the A and H antigen was extensive in duodenum of most pigs, and occurred in jejunum and ileum of some pigs, the VLPs of NV, HS66, MD145, HV, TV only bound to the duodenum and in a similar although less extensive manner to that of the A/H antigen distribution. This suggests that additional factors may be present that permit VLP binding to cells of the duodenum that are lacking in the distal small intestine, as the presence of A and H antigen alone did not guarantee VLP binding. The HS66 VLP binding to only the duodenum of some pigs and not to the jejunum and ileum may be explained by the fact that many pigs expressed HBGA mainly in the duodenum. This limited distribution of VLPs was surprising because in our previous study of the pathogenesis of HuNoV in the Gn pigs (S. Cheetham, M. Souza, T. Meulia, S. Grimes, M.G. Han and L.J. Saif, Abstr. 24th American Society for Virology, abstr W50-8, 2005 and submitted for publication), we observed positive IF in both the duodenum and the jejunum, but only in a few cells in ileum when tested using NS14 MAb (kindly provided by Dr. M. Estes) to the viral capsid of NoV GII strains.
Of relevance to our results for pigs, the *FUT 3* gene, which encodes an enzyme that produces the Le antigens is not present in mammals other than hominids (22). However in our study, HV and TV VLPs that were previously described to bind only to Le<sup>b</sup>, bound to A<sup>+</sup> pig phenotype and to a lesser extent to H<sup>+</sup> pig phenotype intestinal tissues. Thus, the binding of VLPs from these strains must be mediated by the A/H antigens or other molecules in swine. Most mammals express HBGA from the ABO(H) gene family, with some variations (22). Therefore it is possible that pigs also have variations in a Lewis-like gene product. In swine, DSV VLPs bound to H2 antigens in the salivary ducts, but not in the intestine. Prior incubation with MAb to H1 had no effect, whereas incubation with H2 antibody inhibited DSV VLPs from binding to the salivary ducts.

Our blocking assays using MAbs to the A, H1 and H2 antigens showed that blocking was incomplete with either the MAbs used separately or in combination. These results might indicate that the epitope targeted by the MAb is different from the one used by the VLPs for binding or that other molecules are involved. In spite of the extensive data relating the ABO(H) and Se phenotypes with susceptibility or resistance to NoV, it remains unclear whether NoVs utilize HBGA as a primary receptor or a coreceptor (11). In addition, some NoV GII strains (rUEV, rKAV, rCHV) may bind two different receptors, the HBGA and the heparan sulfate proteoglycan (34). Our data of both the distribution of the A/H antigens compared to the distribution of VLP binding (to duodenum) together with the incomplete blocking of the VLP binding by A, H1 or H2 or a combination of these, supports the existence of more than one cell receptor for NoVs.
A recent study showed that Norwalk virus VLPs bound to porcine but not to bovine mucin in a dose dependent manner and that this binding was inhibited by synthetic oligosaccharides and human saliva (37). In that work the mucins from pigs and cattle were not typed but they are glycoproteins carrying HBGA. Probably, the bovine type used was of inadequate phenotype for inhibiting the binding, because calves have \( FUT1 \) and \( FUT2 \) homologs (2) and some calves express A and/or H antigens (identified using MAbs to human A or H antigens) in their intestinal and buccal tissues (Cheetham and Saif, unpublished). Recently it was determined that mucins in human breast milk from Se\(^+\) individuals could inhibit the binding of Norwalk virus VLPs to their carbohydrate ligands (31). Goblet cells contain these mucins. Interestingly goblet cell depletion has been described in the intestine of children with transplants undergoing NoV infections (18, 19, 26). In the children with transplants both osmotic and also secretory diarrhea were described due to NoV infection (18, 19). Similarly, we observed goblet cell depletion in some of the NoV-infected pigs in our pathogenesis study although this event was also variable. However in our pathogenesis study, viral antigen was not detected in goblet cells in the small intestine from Gn pigs by IF and viral particles were not observed by transmission electron microscopy in this cell type. Therefore, goblet cell depletion could represent part of a host innate defense mechanism to remove pathogens and might be regulated by cytokines (7) and not by the direct viral infection of this type of cell.
This type of innate response has also been suggested from a rotavirus study in mice whereby goblet cells and mucins could have an active role in defense by regulation of their quantities and mucin composition (3). More experiments are needed to substantiate the possible role of NoV-related goblet cell depletion and the role of mucins.

In our pathogenesis study, most pigs were of type A+, with fewer H+ type. This may be explained by the fact that all these animals came from the same closed herd of Hampshire breed. The proportion of the A/H phenotypes in the swine population has not been reported. We tested 10 conventional pigs from a different herd with Berkshire and Landrace cross-bred pigs. Our limited information suggests that A/H allelic frequencies may vary depending on the breed and/or herd. In the case of HS66, pigs expressing either A or H phenotypes became infected. This may not be the case for other NoV strains such that determination of the pig’s A/H histo-phenotype would permit matching of the porcine phenotype with the comparable phenotype-specific HuNoV strains. Typing the ABO(H) antigens in human oral squamous epithelium has been performed by immunohistochemistry (27) and it was recently also described in monkeys and pigs (4). Similarly our described typing assay using buccal cells permits A/H typing of live pigs in a fast and simple test.

In addition to the expression of the putative NoV receptors in the pig gastrointestinal tract, the detection of NoV antibodies in swine, with more than 50% of the pigs having antibodies to GI and GII HuNoVs, raises questions about the role of pigs as reservoirs for NoV strains transmissible to humans (8).
The VLPs from a swine NoV strain (SW918) did not bind to human saliva and no data was available on pig saliva binding to human VLPs (8). We are currently testing the VLPs we have available for their binding to pig saliva.

Several animals models were previously evaluated for human NoV replication (9). Only chimpanzees shed Norwalk virus although they were asymptomatic (39). Also 2 of 4 Rhesus monkeys seroconverted and produced soft stools after oral inoculation with Norwalk virus, but fecal virus shedding was not detected (6). At that time molecular techniques were not available; therefore, viral detection was less sensitive. In addition, the role of HBGA in susceptibility and resistance to NoVs was unrecognized so the animals tested could have been of a resistant phenotype or may not have expressed these surface antigens. Most of these experimental animal model studies were done using Norwalk virus, the prototype of NoV GI. In more recent studies where some prosimians and Rhesus macaques were orally inoculated with Norwalk virus, a low level of replication may have occurred in the prosimians, but a high replication level was detected in one Rhesus macaque (30). All animals in the study were Se⁺ and the Rhesus macaque that shed virus in its feces for 19 days was A⁺, whereas the other 2 were B⁺ and H⁺, respectively. The prosimians were A⁺ or AB⁻. In these species the Norwalk virus and Grimsby virus VLP binding assays of human RBC and the HBGA and Se typing results showed no correlation with susceptibility to infection (30). Norwalk virus VLPs did not hemagglutinate Rhesus macaques RBCs whereas Grimsby VLPs did. However, infection of Rhesus macaques occurred with Norwalk virus. Unfortunately, Rhesus macaques were not inoculated with Grimsby virus so no information was available for that strain. Our
finding that Gn pigs that express A/H antigens in their intestines have a higher rate of NoV GII/4 infection (based on seroconversion or diarrhea) is in agreement with what has been determined in humans whereby VLP hemagglutination (or expression of the A/H antigens in RBC) is correlated to susceptibility to Norwalk virus (17).

In summary, our study demonstrated that pigs could be typed for their expression of HBGA in their mucosal tissues and that the expression of A/H antigens in pigs is similar to humans. We also observed that there was complete agreement (except for 1 pig) between the expression of HBGA in the buccal mucosa (determined by IF, IHC or HI) and the intestinal mucosa. Therefore determination of the A/H phenotype is possible prior to NoV inoculation of live pigs. The VLP binding assays corroborated that NoV VLP binding specificities vary among virus genotypes in pig tissues as they do in human tissues and for in vitro binding of synthetic oligosaccharides. In addition, although some VLPs bound as expected (NV and MD145, HS66), HV and TV VLPs surprisingly also bound to pig tissues, suggesting that their binding target may differ in pigs compared to humans. Blocking assays using MAbs to A and H antigens indicated that likely more than one factor is involved in VLP binding to the cell surface, and probably this factor is present in porcine mucin which was capable of totally blocking the VLP binding to tissue slides. Finally, our data further supports use of the Gn pig as a good model to study the pathogenesis of human NoVs, whereby infection outcomes relate to HBGA expression as in humans. To our knowledge this is the first study to compare the HBGA expression in the pig to the NoV infection outcome.
3.6 ACKNOWLEDGMENTS

Thanks to M. Souza, R. McGregor, Drs T. Meulia, Q. Wang and L.J.Saif for their substantial collaboration. I also thank Drs. M. G. Han and M. Azevedo for their suggestions and Drs. S. Monroe, K. Green and M. K. Estes for the reagents they provided. We also thank Dr. J. Hanson and R. McCormick for the animal care and Andrea Kaszas and David Fulton at the Molecular and Cellular Imaging Center, who provided technical assistance. We thank Drs. K. Theil and D. Jackwood for reviewing the manuscript.

Salaries and research support were provided by state and federal funds appropriated to the Ohio Agricultural Research and Development Center, The Ohio State University. This work was supported by the National Institute of Allergies and Infectious Diseases, National Institute of Health Grant # AI49742.
3.7 REFERENCES


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**Table 3.1.** Results of NoV VLP binding and A or H MAb blocking assays done on paraffin sections of pig duodenal and buccal tissues of A+ and H+ Gn pigs

a. Norwalk virus, b. Desert Shield virus, c. Hawaii virus, d. Toronto virus

e. Buccal tissue not done  f. Duodenum g. Monoclonal antibody h. Expected blocking result from reference binding in vitro

i. No blocking  j. Partial blocking k. Not applicable because of no binding. Letters in parenthesis and italics indicate type of Lewis antigen.
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**Table 3.2.** A/H phenotype results for the Gn pigs in the NoV GII/4 pathogenesis study in relation to fecal virus shedding, diarrhea and seroconversion

a. Histo-blood group antigens

b. Defined as = 2 days of RT-PCR + rectal swab

c. Defined as = 2 days of diarrhea

d. Tested by IgG antibody ELISA using NoV GII/4 VLPs as antigen

e. Differed statistically (p< 0.05) A and H phenotypes were compared to each other and A/H together were compared to non A/H

Figure 3.1 Schematic of hemagglutination inhibition test using buccal cells to determine the A/H phenotype of live pigs
Figure 3.2. Immunohistochemistry for A/H typing of a duodenal section from a Gn pig:
A) MAb to type A reacted with goblet cells (arrow heads), the surface of some enterocytes (white arrows), Brunner glands (black arrows). B) Higher magnification of the same slide with detail of reactive goblet cells and enterocytes. C) Same tissue did not react with MAb to H1. Bars A=50 µm B=25 µm C=150 µm
Figure 3.3  IF for A/H phenotyping: A-F) A+ pig phenotype; G-I) H+ pig phenotype.
A) Duodenum of an A+ pig with MAb to type A showed signal in goblet cells (arrow head), surface of enterocytes (white arrow) some crypts (thick arrow). B) Same A+ pig with MAb to H1 showed signal in some surface enterocytes (white arrow) but not in goblet cells or crypts. C) Same A+ pig with MAb to H2, showed localized reaction with enterocytes (white arrow) and a few crypt cells stained (thick arrow). D) Jejunum of the same A+ pig with MAb to type A showing fewer reactive cells E) Ileum of the same A+ pig with MAb to type A showed very little positive specific reaction and more background. F) Duodenum of a different A+ pig with MAb to H2 showed reaction in Brunner glands (black arrow). G) Duodenum of an H+ pig with MAb to type A showed no reaction. H) Duodenum from the same H+ pig with MAb to H1, showed no reaction. I) Duodenum from the same H+ pig with MAb to H2 showed reactive goblet cells (arrow head), surface of enterocytes (white arrow) and some crypt cells (thick arrow). Bars = 100μm
Figure 3.4 IF of VLP binding in duodenum and buccal tissues.
Figure 3.4. IF of VLP binding in duodenum and buccal tissues: in the intestinal tissues, arrow head denotes goblet cells; white arrow denotes surface of enterocytes; thick arrow denotes crypts and black arrow denotes Brunner glands. In the buccal tissues, thin arrow denotes spinous cell layer; white pointer denotes spinous parabasal cell layer transition; black arrow denotes buccal salivary glands and white arrow denotes salivary ducts. A) A+ pig with NV (GI/1) VLPs that bound to some goblet cells (arrow head) and surface of some enterocytes (white arrow) and minimally to crypts (thick arrow). B) H+ pig with NV VLPs that bound to some goblet cells (arrow head) and extensively to enterocyte surfaces (white arrow) as well as Brunner glands (black arrow). C) Parakeratinized stratified squamous epithelium of the oral mucosa (buccal tissue) from an A+ pig, MAb to A antigen reacted with spinous layer (thin arrow). D) Buccal tissue from a H+ pig reacted with MAb to type H2 deeper in the parabasal cell layer (white pointer). E) A+ pig with HV (GII/1) VLPs reacted within certain areas of the intestinal section where signal was observed in some goblet cells (arrow head) and enterocytes (white arrow). F) H+ pig with HV VLPs showing reactivity in enterocytes (white arrow) but not with goblet cells. G) NV VLPs bound to A+ pig phenotype buccal tissue (that also expresses H2) at the spinous layer (thin arrow). H) NV VLPs bound to H+ pig phenotype buccal tissue, deeper at the spinous parabasal layer transition (white pointer). I) A+ pig with TV (GII/3) VLPs bound in a few locations to some enterocytes (white arrow). J) H+ pig with TV VLPs showing more extensive binding to enterocytes (white arrow). K) MD145 VLPs (as well as HS66) bound to A+ pig phenotype (also expressing H2) at the superficial spinous cell layer (thin arrow). L) MD145 VLPs (as well as HS66) bound to H+ pig phenotype deeper and less extensively (white pointer). M) A+ pig with MD145 (GII/4) VLPs as well as
HS66 (not shown) bound to scattered enterocytes (white arrow) and Brunner glands (black arrow). N) H+ pig with MD145 (same for HS66) VLPs bound more extensively with enterocytes (white arrow), few goblet cells (arrow head) and Brunner glands (black arrow). O) MD145 (as well as HS66) VLPs bound to buccal glands (black arrow) and duct linings (white arrow) from H+ pigs. P) DSV VLPs did not bind to buccal epithelium from an H+ pig. Q) A+ pig with MD145 VLPs binding to goblet cells (arrow head). R) H+ pig with MD145 VLPs binding to the surface of enterocytes (white arrow). S) Buccal glands (black arrow) and duct lining (white arrow) from an H+ pig reacted with MAb to H2 antigen. T) DSV VLPs bound to duct lining from a H+ pig (white arrow). Bars A-P, S-T =100 µm Q=10 µm R=50 µm
CHAPTER 4

CASE REPORT: EVALUATION OF NOROVIRUS SHEDDING BY REAL-TIME RT-PCR IN FECAL SAMPLES COLLECTED DURING A TWELVE-HOUR PERIOD FROM AN ACUTE CASE OF HUMAN GASTROENTERITIS AND EVALUATION OF REAL-TIME RT-PCR FOR APPLICATION TO RECTAL SWAB SAMPLES OF HUMAN NOROVIRUS INFECTED PIGS

4.1 SUMMARY

We studied human NoV shedding in fecal samples collected at various time points from a human case during an acute case of gastroenteritis. The NoV GII/4 viral shedding varied significantly, reaching 10,000 and 20-fold increases by Real-time RT-PCR (SYBR Green) and antigen ELISA respectively, during the 12 h period studied. When Real-time RT-PCR was applied to rectal swab dilutions from pigs inoculated with a HuNoV GII/4 strain the quantification was not possible because fecal virus shedding was too low. By antigen ELISA viral protein was detected in the intestinal contents (IC) (Chapter 1) but very rarely in the rectal swab fluids.
4.2 INTRODUCTION

Human Enteric Caliciviruses (HECV) are the most common agents causing non-bacterial gastroenteritis worldwide. They are classified into two genera Norovirus (NoV) and Sapovirus (SaV) and further subdivided into genogroups (G) and clusters (1). The NoV GII cluster 4 (GII/4) strains have been identified as the most common circulating strains presently in several continents. Generally, symptoms of NoV gastroenteritis last 24-48 hrs and are self-limiting (14). Recent human volunteer studies performed with more sensitive molecular techniques for detection of GI and GII NoV strains have provided additional information. These studies as well as case reports have described aspects of their pathogenesis such as host susceptibility and/or resistance phenotype, asymptomatic shedding, long periods of shedding after symptoms ceased and long symptomatic disease in immunosuppressed individuals (5, 7, 8, 13).

The objective of this study was to gather information on the early dynamics of in vivo NoV infection needed to understand the replication and shedding of these viruses in the early hours after acute disease. We detected NoV in diarrheic fecal samples from a human case with acute gastroenteritis by RT-PCR using NoV GI and GII-specific primers and sequenced the PCR products. We estimated the amount of NoV shedding by semi-quantitative Real-time RT-PCR (SYBR Green I). This technique has been previously validated (6, 9, 11, 12). We also measured and compared the amount of viral capsid protein in the fecal samples by antigen-ELISA and evaluated seroconversion by IgG antibody-ELISA to homologous and heterologous NoV VLPs. We also tested the application of this technique to the quantification of fecal viral shedding by HuNoV-inoculated Gn Pigs.
4.3 MATERIALS AND METHODS

Six fecal samples from a human case with symptoms of acute gastroenteritis were collected. The individual was a young adult female presenting A\(^+\) Se\(^+\) histo-blood phenotype with no other significant medical history. The virus dose was unknown and although not precisely known, incubation time was estimated as 18 hrs. Diarrhea and abdominal cramps were the predominant signs that started at 4 AM and continued for approximately 18 hrs. Weakness was manifested for several days. Fecal samples were collected consecutively every 1-3 hrs starting at 6 AM and finishing at 6 PM.

Total RNA was extracted from the fecal samples using Trizol (Invitrogen) according to the manufacturer’s instructions. RT-PCR using Mon 431/33 (for NoV GII) and 432/34 (for NoV GI) primers (10) (Figure 4.1) and subsequent sequencing of the 211bp RT-PCR product amplified from the RdRp region, permitted identification of the strain as a NoV GII/4. Semiquantitative Real-time RT-PCR with SYBR Green was performed as described by Pang (9) with several modifications. Specifically, we performed a one step Real-time RT-PCR (Roche, SYBR green I) instead of two step using the Mon431/433 primer pair. Viral shedding was calculated using serial dilutions of a competitive RT-PCR RNA internal control designed in our lab for the routine screening of samples for both NoV and SaV by conventional RT-PCR (Figure 1). This internal control can be used in separate tubes as an external standard curve for the Real-time RT-PCR (Figure 4.3.1). Briefly, for the construction of the internal control we amplified 284 bp from the \(\beta2\) Microglobulin constitutive gene with primers containing hanging tails coding for the Mon and P289/290 primer sequences (3) so it could be used
in routine screening of samples for both SaV and NoV. The 360bp amplified product was gel purified and cloned into a TA cloning vector (Invitrogen) in which after linearization, it was in vitro transcribed into RNA using T7 RiboMAX (Promega).

We designed the internal control product to be larger than the target product because smaller products are preferentially amplified when competition occurs (Figure 4.1).

Others have employed cDNA NoV sequences as external standard when using a 2 step reaction (see Chapter 1). We believe RNA is more appropriate in our case because of the one step reaction that we chose because it is simpler and avoids cross contamination and carryover contamination.

Once the reaction was standardized, RNA extracted from daily-collected rectal swab fluids of two Gn pigs inoculated with a HuNoV from GII/4 (see Chapter 2) were tested.

4.4 RESULTS

Viral shedding levels differed significantly throughout the 12 h period. Different time points tested by each method, one measuring viral genome and the other viral antigen are shown in Figure 4.2. Both techniques showed a curve of similar trend and detected a peak of viral shedding in the early afternoon with the capsid protein curve slightly shifted to the right as compared to the one of Real-time RT-PCR suggesting a subtle delay in the capsid expression. A 100-fold increase in antigen titer (from 100 to 10,000) by antigen-ELISA and a 5.5-log increase in the RNA copy numbers (from 5 x 10^7 to 10^{13}) by Real-time RT-PCR were detected (Table 4.1 and Figure 4.4)
The fluorescence measurement was semiquantitative using the arithmetic fit points method (Figure 4.4). The external standard copy number was calculated by multiplying the RNA concentration measured by the emitted fluorescence multiplied by the estimated number of copies/ng. The latter was calculated by dividing Avogadro’s number by the multiplication of the length of the amplified product and by 340g/mol (mass of RNA). This information and the corresponding dilutions were entered into the light cycler program. This formula also corrects for variation in fluorescence due to differences in size between the internal control (320 bp) and the viral amplification product (211 bp). The genome copy number was then calculated by the program based on their crossing points to the regression line of the standard curve (Table 4.3). This curve is a linear regression of serial dilutions of known concentrations of the external standard. The fluorescence from primer dimer formations (Figure 4.5) or primer gymnastics previously described for these primers could be due to the difference in melting temperature (TM) between primers (53 and 63°C) and it could not be overcome by increasing stringency or by measuring fluorescence at a temperature between the primer dimers and the products melting points (10). We believe that this effect does not compromise our results since it occurred equally for all samples and did not alter the proportional differences among them. Also because we used the arithmetic baseline adjustment and the Fit points method to quantify which are indicated for low copy numbers and high background situations. The threshold cycle, the point at which the fluorescence rises appreciably above the background signal, was set accordingly to minimize the interference from primer dimers.
Sandwich antigen (Ag) ELISA was used to measure the amount of virus antigen (protein) in human feces, using VLPs, guinea pig and rabbit hyperimmune antisera to GII/4. Seroconversion was evaluated by IgG antibody ELISA with acute serum from day 4 post disease onset and convalescent serum from day 21. Seroconversion to NoV GII/4 was detected with an 8-15-fold increase in specific antibody titer between the acute and convalescent phase sera. Seroconversion (4-fold increase) was also evident against GII/3 VLPs, but not against GII/1 and GI/3 VLPs (Table 4.2).

When rectal swab fluids from HuNoV-inoculated pigs were tested by Real-time RT-PCR the viral quantification result was unreliable using the external standard curve (Figure 4.6). Primer dimers appeared in higher proportions probably because of low viral copy number (Figure 4.7). Amplification was specific as observed by localization of the peaks from the viral amplicons at the right melting point (83.5 ° C). However, only amplicons from PID 1 and 2 could be quantified (Figure 4.8), indicating that the Real-time RT-PCR reaction used was less sensitive than traditional RT-PCR followed by microwell hybridization. When the same samples were tested by RT-PCR followed by microwell hybridization, viral shedding in rectal swab fluids was detected until PID 6 (see Chapter 2).

4.5 DISCUSSION

Although the pathogenesis of a murine NoV has been reported, the pathogenesis of the human NoV is not yet well understood and the clinical signs caused by these two groups of NoV differ significantly, being systemic and enteric, respectively (4). To date, no enteric disease animal model is available for human NoV. Volunteer studies have
provided valuable daily shedding information but, NoV shedding within the first 24 hrs post challenge has not been examined. This is the first report characterizing the short term human NoV shedding in an infected individual. The magnitude of increase was highest ten hours after the onset of diarrhea and then decreases notably. Hohne et al.(2) suggested that sample collection time after onset of illness and individual immune responses could be responsible for the broad virus load detected. In this same study Hohne et al. presented the viral shedding quantification in a patient sampled in the acute phase and after symptoms disappeared (days 0, 3, 22, 31, 48, 60 and 87). Surprisingly, the virus shedding in that patient was maintained at similar levels in both the disease and recovery phase. Unfortunately, in our case we did not have fecal samples available from this case to continue the observations of viral shedding after the diarrhea ceased to determine if there were other peaks of shedding or the duration and quantification of shedding in the convalescent phase. Generalizations cannot be made from this single case investigated because of the individual genetic factors. Nevertheless, this data shows that in this case there was a peak of maximum shedding after the first round of viral replication ten hours after the onset of symptoms. The \textit{in vivo} study during the first hours post infection is essential to understand the pathogenesis of NoV because of the acute nature of this disease.

The evaluation of this Real-time RT-PCR for fecal viral shedding in rectal swab fluids collected from HuNoV-inoculated Gn pigs indicated that this quantification method needs to be optimized for low copy numbers. The low viral shedding was not surprising because the samples were diluted rectal swab fluids as compared to the human samples, that were stools. In addition, viral replication was not as efficient in swine as it
is in humans based on the viral titers shed and the failure to detect virus in most IC samples by IEM. When testing Gn pig IC by Real-time RT-PCR, in some IC samples the presence of inhibitors did not allow the reaction to occur, whereas other IC contained virus below the quantification range. Further adaptation of the virus to swine or the design of new primers or reaction conditions may improve the reliability for the quantification. At this point the conclusion is that the specificity of the products can be determined, and the relative quantification between samples can be deduced.

4.6 ACKNOWLEDGMENTS

Thanks to Menira Souza for her technical collaboration and to Dr. Juliette Hanson and Richard Mc Cormick for the animal care. We thank Dr. D. Jackwood and Dr. S. Sreevatsan for their technical assistance and discussions and Dr. K. Theil for reviewing this manuscript. We also thank Dr. K.Green (NIH) for kindly providing virus-like particles, guinea pig and rabbit hyperimmune antiserum to GII/4.
4.7 REFERENCES


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<td>Acute serum</td>
<td>1,000</td>
</tr>
<tr>
<td>Convalescent serum</td>
<td>15,000</td>
</tr>
</tbody>
</table>

Table 4.2. Evaluation of seroconversion by IgG antibody-ELISA using homologous (GII/4) VLPs and heterologous human NoV VLPs.
Figure 4.1 RT-PCR products (Mon 431/33). Fecal samples (lanes 1-6), negative control (lane 7), positive control (lane 8), molecular weight ladder (lane 9). 2% agarose gel stained with ethidium bromide. IC: internal control.

Forward primer to create IC:
5’-GATTACTCCAAGTGCACTCCACTGGACCAGTGGCCCTAATCACTGCTCTCAGTGCTG-3’

Reverse primer to create IC:
5’-TGCAATTAATCACCATAAGAAATCCATCCAGAATGATCGAGATCGCTGCT-3’

Red tail : 289/290       Blue tail: Mon       Black: $\beta_2$ Microglobulin
Figure 4.2 Detection of fecal viral shedding by real-time RT-PCR and antigen ELISA
Figure 4.3 External standard curve constructed with serial dilutions of known concentrations of the internal control.
Figure 4.4 Quantification of HuNoV RNA by arithmetic fit point method.
Figure 4.5 Melting points of the viral and external standard amplicons
Figure 4.6 External standard curve for the quantification of HuNoV viral RNA in Gn pigs rectal swab fluids.

Figure 4.7 Melting points of primer dimers, viral product in rectal swab fluids of Gn pigs inoculated with HuNoV GII/4 and external standard amplicons. PID: post-inoculation day.
Figure 4.8 Quantification of viral RNA from rectal swab fluids from Gn pigs inoculated with HuNoV GII/4 calculated by the arithmetic Fit points method.

Samples 1-7: pig 3 post inoculation day1 and successive days. Sample 8: inoculum.
Sample 9: positive control of the RT-PCR reaction. Sample 10: negative control.
Samples 11-14: serial dilutions of the internal control used as an external standard.
CHAPTER 5

GENERAL CONCLUSIONS

5.1 SUMMARY

Noroviruses are a public health concern. In addition to the economic repercussions of gastroenteritis outbreaks and the impact of diarrhea episodes on the general population, there are more than 4,000 deaths annually due to gastroenteritis in the U.S. (2). In this country over 250 million episodes of diarrhea result in approximately 450,000 adults and 160,000 children being hospitalized each year (2). Noroviruses are the leading cause of non bacterial gastroenteritis in the U.S. (as well as many other countries), with more than ninety percent of the outbreaks attributed to these viruses. Although NoV infections occur in both developed and developing countries, the death toll in the later could be much higher but little information is available on the loss of human lives due to NoV in developing countries.

The epidemiology of NoV infections may be as a result of human actions as suggested by the identification of pandemic strains (1). This may reflect globalization of trade resulting in goods and people traveling across continents. Also clinical characteristics have changed for NoVs. They can be acute episodes in otherwise healthy people, chronic diarrhea in transplant recipients or a more life threatening disease with
cases of intravascular coagulation syndrome in adults under physical stress (1). Moreover the identification of NoVs in swine and cattle that are genetically closely related to the human strains are worrisome for their zoonotic potential (4).

Despite arduous research for more than 3 decades and numerous studies of this group of viruses, our understanding of NoV biology and pathogenesis is limited. *In vitro* studies performed with NoV replicons or cell culture surrogates such as FCV and the recently discovered MNV-1 have provided useful information. However, the existence of an animal model is needed because human trials are difficult, costly and restricted. In recent human studies, the immune responses to potential mucosal vaccines (VLPs) were evaluated but lacking NoV challenge the protective efficacy of the immune responses was not measured. In addition to examining intestinal correlates of immune protection, the intestinal responses measured by cytokine profiles, evaluation of cytokine secreting cells and antibody secreting cells to define the types of local immune responses is less feasible in humans. The recent use of monkeys in NoV research has given promising results. Nevertheless, because of their costs and ethical reasons these experiments have been limited to a few animals and intestinal responses have not been evaluated, making this model impractical for vaccine evaluation.

Gnotobiotic pigs have prove to be good models to study other viruses that cause gastroenteritis in humans and to test vaccines (3). For this reason, the evaluation of Gn pigs for human NoV replication was undertaken. Development of a much needed animal model for NoV disease should have a dramatic impact on future research. In Chapter 2 we demonstrate that a human NoV strain from GII/4 can replicate in Gn pigs. This has two important consequences; first, Gn pigs can be used as a model to study the
pathogenesis and biology of NoVs, host responses to NoV infection and test vaccines; and second, it demonstrates that pigs may be either reservoirs for human NoVs or could be animal hosts in which new human strains, resulting from recombination between human and swine strains could emerge. A potential recombination event could be further studied in this model because Gn pigs are confined to isolator units and a mixed infection with human and swine origin strains could be evaluated for the emergence of recombinants without the risk of releasing new stains into the environment.

The observation that some pigs are refractory to infection by the human NoV strain tested is an indication that the Gn pig model mimics humans, where differential susceptibility due to genetic factors has been extensively described. The results from the studies in Chapter 3 imply that in swine, similar factors are responsible for their resistance. On the other hand, these findings highlight a need to circumvent this resistance when developing a vaccine study. To evaluate protection rates of a NoV vaccine in the Gn pig model, the following could be tried in further studies: identify resistant pigs (to be excluded), identify a NoV strain that causes disease in 100% of the animals or increase disease rates by other means such as immunosuppression. These alternatives are currently being evaluated. Application of new technology to NoV studies such as microarrays will also provide information that will direct future studies and confirm preliminary results. Currently, the Gn pig intestinal response to human NoV infection is being evaluated by using a 70 mer oligoarray comprising 13 thousand genes from swine. Hopefully, this will help to determine the unique pathways involved in the pathogenesis of NoVs, the occurrence of apoptosis, the role of innate immunity and resolve the factors required for shaping adaptive immunity to NoV infection.
Although widespread and economically important, the major impact of NoV disease is limited to highly vulnerable groups such as the elderly and immunosuppressed. Therefore, NoVs receive much less attention than other virus threats such as human immunodeficiency virus, influenza virus or hepatitis C virus. However, it is important to protect those groups most at risk and to be prepared for future strains that may emerge as a result of the continued evolution of NoV genomes since as RNA viruses they have tremendous flexibility which makes them a continuing potential disease threat.
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