LEENA HUHTI

Significance of the Norovirus GII.4 Genotype as a Cause of Acute Gastroenteritis in Finnish Children, and the Production of a Candidate Virus-like Particle Vaccine

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ACADEMIC DISSERTATION
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UNIVERSITY OF TAMPERE

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To my family,

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List of Original Communications

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I. Puustinen L, Blazevic V, Huhti L, Szakal ED, Halkosalo A, Salminen M, Vesikari T. Norovirus genotypes in endemic acute gastroenteritis of infants and children in Finland between 1994 and 2007. Epidemiology and Infection. 2012 Feb; 140(2):268-75.

II. Huhti L, Szakal ED, Puustinen L, Salminen M, Huhtala H, Valve O, Blazevic V, Vesikari T. Norovirus GII-4 causes a more severe gastroenteritis than other noroviruses in young children. Journal of Infectious Diseases. 2011 May 15; 203(10):1442-4.

III. Huhti L, Blazevic V, Puustinen L, Hemming M, Salminen M, Vesikari T. Genetic analyses of norovirus GII.4 variants in Finnish children from 1998 to 2013. Infection, Genetics and Evolution. 2014 Aug; 26:65-71.

IV. Huhti L, Blazevic V, Nurminen K, Koho T, Hytönen VP, Vesikari T. A comparison of methods for purification and concentration of norovirus GII-4 capsid virus-like particles. Archives of Virology. 2010 Nov; 155(11):1855-8.

V. Huhti L, Tamminen K, Vesikari T, Blazevic V. Characterization and immunogenicity of norovirus capsid-derived virus-like particles purified by anion exchange chromatography. Archives of Virology. 2013 May; 158(5):933-42

Abbreviations

3CLpro	3C-like protease
aa	Amino acid
Ab	Antibody
AGE	Acute gastroenteritis
BES	Baculovirus expression system
bp	Basepair
BV	Baculovirus
cDNA	Complimentary deoxyribonucleic acid
CsCl	Cesium chloride
dNTP	Deoxyribonucleoside triphosphate
dpi	Days post-infection
dsDNA	Double-stranded deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscopy
FBVE	Foodborne Viruses in Europe Network
FUT	Fucosyltransferase
G	Genogroup
gp	Glycoprotein
HBGA	Histo-blood group antigen
HRP	Horseradish peroxidase
IEM	Immune electron microscopy
Ig	Immunoglobulin
LAL	Limulus amebocyte lysate
MOI	Multiplicity of infection
mRNA	Messenger RNA
MuNoV	Murine norovirus
NIP	National Immunization Programme
NLV	Norwalk-like virus
NoV	Norovirus
NS	Non-structural

nt	Nucleotide
NTPase	Nucleoside triphosphate
OD	Optical densities
OPD	O-Phenylenediamide Dihydrochloride
ORF	Open reading frame
P	Protruding
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
poly-A	Polyadenylated
RdRp	RNA-dependent RNA polymerase
RRV-TV	Rhesus-human reassortant rotavirus tetravalent vaccine
RT	Room temperature
RT-PCR	Real-time polymerase chain reaction
RV	Rotavirus
S	Shell
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Sf	Spodoptera frugiperda
SLV	Sapporo-like virus
SRSV	Small round structured virus
TBS	Tris-buffered saline
Th	T helper cell
UTR	Untranslated region
VLP	Virus-like particle
VPg	Viral protein genome-linked

Abstract

Norovirus (NoV) is the leading causative agent of acute gastroenteritis (AGE) outbreaks among all age groups, and it is the second most common viral agent after rotavirus (RV) to cause clinically severe gastroenteritis in young children. After the introduction of a RV vaccine into the National Immunization Program of Finland in 2009, NoV has become the leading cause of AGE in children. Since the 1990s, NoV GII.4 has been the predominant genotype circulating worldwide in outbreaks and in sporadic gastroenteritis in children, with new variants emerging approximately every two to three years. For the prevention of NoV infections, NoV vaccines based on virus-like particles (VLPs) are being developed. A vaccine against NoV GII.4 is urgently needed.

This dissertation investigates the prevalence of NoV genotypes, and the antigenic changes and clinical features of NoV GII.4 in Finnish children for the general purpose of VLP-based candidate vaccine production and evaluation. The clinical study material is composed of two parts. First, fecal specimens were collected from AGE cases in children who participated in RV vaccine efficacy trials in Finland from 1993 to 2007. Second, fecal specimens were collected from children who were treated at Tampere University Hospital because of AGE from 2006 to 2013.

The distribution of NoV genotypes was first studied in sporadic NoV AGE cases among children in the community between the years 1993 and 2007. NoV GII.4 was the most common genotype (46%) and it predominanted for three seasons (1998–1999, 2002–2003, 2006–2007). Secondly, in children treated at Tampere University Hospital, NoV AGE cases were mainly caused by NoV GII.4 (79%). Altogether, NoV GII.4 was found in 55% of non-outbreak NoV cases in children during the entire period of 1998–2013, with a new variant of GII.4 appearing every two to three years. Six different GII.4 variants were detected, and these coincided with NoV epidemic years. In addition, GII.4 was found to cause more severe cases of gastroenteritis than other NoV genotypes.

For the purpose of vaccine development, NoV GII.4 VLPs were produced using the baculovirus expression system in Sf9 insect cells. The VLPs were purified with several purification procedures and the purity, yield, morphology, and functionality of the NoV VLPs were assessed. To obtain high quality NoV VLPs, two

discontinuous sucrose gradient centrifugations were used. However, the density gradient-purified VLP preparations contained impurities from the expression vector. It was thus necessary to purify NoV VLPs further. Chromatographically purified NoV VLP preparations were devoid of impurities and maintained the icosahedral capsid structure. The purified VLPs induced efficient NoV GII.4-specific immune responses in mice.

Antigenic variations in the P2 domain of GII.4 major antigenic epitopes, known as blockade epitopes, are linked to NoV binding profile of human histo-blood group antigens. Genetic analyses of predominant NoV GII.4 variants that circulated in Finland between 1998 and 2013 showed that NoV GII.4 undergo significant variation in the major antigenic epitopes on the surface of the capsid P2 domain. These findings were similar to those seen previously in NoV outbreaks in other populations. Future NoV vaccines should induce either type-specific immunity for each variant – in which case the vaccine would need to be frequently reformulated – or produce broadly cross-protective immunity covering multiple variants. Promisingly, the candidate NoV GII.4 VLP vaccine, based on a 1999 NoV isolate produced in our laboratory appears to induce a broad spectrum immune response in mice covering the new strains of GII.4 up to 2012.

Tiivistelmä

Norovirus on yleisin virusperäisten ripulitautiepidemioiden aiheuttaja kaikissa ikäryhmissä ja rotaviruksen jälkeen toiseksi yleisin vakavan ripulitaudin aiheuttaja lapsilla. Rotavirusrokote lisättiin Suomen kansalliseen rokotusohjelmaan vuonna 2009, minkä jälkeen rotaviruksen aiheuttamat ripulitaudit ovat merkittävästi vähentyneet. Tämän seurauksena noroviruksista on tullut yleisin ripulitaudin aiheuttaja lapsilla. 1990-luvulta lähtien noroviruksen GII.4-genotyyppi on aiheuttanut suurimman osan noroviruksen aiheuttamista ripulitautiepidemioista, sillä GII.4-genotyypistä kehittyy uusia variantteja muutaman vuoden välein. Norovirusta vastaan ei ole rokotetta, mutta viruksen kaltaisten partikkelien (virus-like particle, VLP) käyttöä norovirusrokotteena arvioidaan parhaillaan. Rokotteen tulisi estää etenkin GII.4-genotyypin aiheuttamat ripuli- ja oksennustaudit.

Tässä työssä tutkittiin norovirusgenotyypin GII.4 esiintyvyyttä, antigeenistä vaihtelua ja kliinistä taudinkuvaa sekä näiden tutkimustulosten perusteella päämääränä oli tuottaa VLP-norovirusrokotekandidaatti. Kliininen tutkimusmateriaali koostui kahdesta osasta: (1) Rotavirusrokotetutkimuksiin vuosina 1993–2007 osallistuneet lapset, joita seurattiin prospektiivisesti ja joiden kaikki esiintyneet ripulitautitapaukset tutkittiin ja (2) vuosina 2006–2013 Tampereen yliopistollisessa sairaalassa ripulitaudin vuoksi hoidetut lapset. Nämä sairaalahoitoiset lasten ripulitaudit olivat keskimäärin vaikeampia kuin prospektiivisessa seurannassa olleiden lasten ripulitaudit.

Vuosien 1993–2007 aikana genotyyppi GII.4 oli yleisin norovirusgenotyyppi (46 %) ja se aiheutti kolme epidemiakautta: vuosina 1998–1999, 2002–2003 ja 2006–2007. Vuosina 2006–2013 GII.4-genotyyppi havaittiin 79 %:ssa noroviruksen aiheuttamista ripulitautitapauksista lapsilla, jotka kävivät ripulitaudin takia Tampereen yliopistollisessa sairaalassa. GII.4 oli vallitsevin genotyyppi (55 %) kaikista Suomessa vuosina 1998–2013 tutkituista lasten ripulitautitapauksista. Tutkimusvuosilta 1998–2013 löydettiin kuusi erilaista GII.4-varianttia. Lisäksi GII.4-genotyypin todettiin aiheuttavan lapsilla keskimäärin vakavamman ripulitaudin kuin muut genotyypit.

Norovirus GII.4-genotyypin kannasta vuodelta 1999 tuotettiin VLP-rokotekandidaatti käyttäen bakulovirus-tuotantosysteemiä hyönteissoluissa. VLP:t

puhdistettiin eri menetelmin ja niiden puhtaus, morfologia, saanto ja immunogeenisyys tutkittiin. Perinteisellä sukroosigradienttisentrifugointiin perustuvalla puhdistusmenetelmällä saatiin parhaat tulokset. Koska tällä menetelmällä puhdistetut VLP-näytteet kuitenkin sisälsivät ekspressiovektorista peräisin olevia epäpuhtauksia, rokotemateriaalia puhdistettiin lisää kromatografialla. Näin puhdistetut VLP-näytteet eivät enää sisältäneet epäpuhtauksia ja säilyttivät ehjän kuorirakenteen. Hiirikokeissa kromatografisesti puhdistetut VLP:t saivat aikaan hyvän GII.4-spesifisen immuunivasteen.

GII.4-genotyypin viruskapsidin kuorirakenteen P2-domeenin antigeenimuutokset tapahtuvat etenkin epitoopeissa, joiden välityksellä norovirus tarttuu ihmisen kudosveriryhmäantigeeneihin. Suomessa vuosina 1998–2013 esiintyneiden GII.4-varianttien P2-domeenin aminohappoketjuja verrattiin keskenään ja GII.4-rokotekandidaatin vastaavaan aminohappoketjuun. Rokotekandidaatin ja GII.4-varianttien välillä havaittiin palautumattomia aminohappomuutoksia. Lasten GII.4-varianteista löydetyt muutokset ovat samankaltaisia aiemmin norovirusepidemioista julkaistujen havaintojen kanssa. Tulevaisuudessa norovirusrokotteen tulisi saada aikaan joko tyyppispesifinen immuniteetti kaikille GII.4-varianteille, jolloin rokote täytyisi usein muokata uudelleen, tai rokotteen tulisi tuottaa laaja-alainen ristiin reagoiva immuniteetti, joka suojaisi monilta eri norovirusgenotyypeiltä ja -varianteilta. Lupaavia tuloksia on julkaistu kehittämästämme GII.4 VLP-rokotekandidaatista, joka on saanut aikaan laaja-alaisen immuunivasteen hiirissä.

Introduction

Noroviruses (NoVs) are the most common viral agents to cause outbreaks of acute gastroenteritis (AGE) worldwide in all age groups, and also the second most common cause of clinically severe gastroenteritis in children after rotavirus (RV) (1,2). Worldwide, NoV causes significant mortality, especially in developing countries: it has been estimated that NoV infections annually lead to a more than 200,000 deaths in children under five years of age in developing countries, 64,000 episodes of diarrhea that require hospitalization, and approximately 900,000 clinic visits in industrialized countries (1). NoV outbreaks have been studied more frequently than sporadic NoV AGE cases in children. In outbreak situations, usually only a small number of stool specimens are examined for NoV diagnosis, whereas in epidemiological studies in children, specimens from every patient must be investigated. The finding that NoV is a common cause of sporadic (non-outbreak) cases of AGE in young children was first reported in Tampere, Finland (3). This was possible because of access to prospectively followed AGE material from a RV vaccine (Rhesus-human reassortant rotavirus tetravalent vaccine, RRV-TV) trial and the availability of the newly described "Le Guyader primers" (4) for NoVs. The study revealed that 20% of children experienced NoV AGE between the ages of 2 and 23 months (3). Following the introduction of the RV vaccination, NoVs have become the leading cause of AGE in children in many countries (5-8).

NoV is transmitted from person-to-person or through contaminated food and water. NoV transmission is facilitated by the high virus particle shedding by NoV infected persons, the low dose needed for infection, the stability of virus particles in the environment, a lack of protective immunity, and rapid virus evolution (2). NoV epidemiology is influenced by many factors, such as individual immune response, population immunity, virus evolution, the environment, and seasonality (2,9).

In 1972, NoV particles were first visualized by immune electron microscopy (IEM) (10). Since then, better molecular diagnostic methods, such as the real-time polymerase chain reaction (RT-PCR), have been employed for better virus detection and more specific genetic analyses (11,12). First detections of NoV using RT-PCR required many primer pairs and the assay did not always detect all NoVs (11). Primer

selection has been challenging for NoV diagnosis. Recently, NoV researchers have published a standardized classification and nomenclature system for NoVs (13,14).

NoVs are divided into six genogroups (GI–GVI) (14-16). Genogroups GI, GII and GIV are known to infect humans. However, genogroup GII is the leading cause of human NoV infections. (16) Since 1995, NoV GII.4 has been the predominant and the most studied genotype; it causes up to 80% of NoV outbreaks and severe sporadic AGE in children (6,17-19). New variants of the GII.4 genotype emerge every two to three years. The rapid genetic variability of GII.4 is based on recombination and point mutations. A novel variant replaces the earlier variant without influence on the persistence of other genotypes. (20-23)

The burden of NoV disease is increasing, and better preventive means are needed. NoV cannot be grown in cell culture, so a live attenuated NoV or an inactivated whole virus vaccine is not feasible. Virus-like particle- (VLP) based vaccines are, in general, immunogenic and safe, and some VLP-based vaccines have already been commercialized (e.g hepatitis B and human papilloma virus) (24-26). NoV capsid protein produced in the baculovirus expression system (BES) in insect cells self-assembles to VLPs. The structure of VLPs mimic the native virus structure and therefore VLPs act like native virions but lack genetic material. NoV VLPs bind to human histo-blood group antigens (HBGAs) which occur on the surface of epithelial cells and in mucosal secretions. VLPs stimulate both humoral and cellular immune systems. (27-29) One NoV VLP vaccine based on GII.4 consensus sequences is already in clinical trials (30). However, the fast genetic evolution of the NoV capsid gene may complicate NoV vaccine development. Our goal has been to include NoV VLPs from GI and GII genogroups into the candidate vaccine. Sequences of GI and GII NoV genotypes isolated from from Finnish patients were initially included into the vaccine, with the aim of producing a broadly reactive immune response that would protect against multiple genotypes and variants of GI and GII NoVs (31).

1 Review of the literature

1.1 Characterization of human noroviruses

1.1.1 History of noroviruses

The first description of the symptoms caused by calicivirus, most probably NoV gastroenteritis, can be associated with Zahorsky's publication in medical literature in 1929; Zahorsky described gastroenteritis as a winter vomiting disease (32). Over twenty years later, scientists found that the causative agent of such an intestinal infection was likely a virus (33,34). In 1968 in Norwalk, Ohio, students and teachers suffered an illness characterized by nausea, vomiting, and abdominal cramps (35). All attempts to culture the causative agent, transmit the agent to an animal were unsuccessful but the properties of the Norwalk agent were already demonstrated: small, heat-stable and non-enveloped virus (36,37). In 1972, Kapikian et al. first identified NoV particles by IEM from stool specimens from the outbreak (10).

Since then, other viral agents that cause gastroenteritis, mainly RV and adenovirus, have been detected by elelctron microscopy (EM). The caliciviral agent of gastroenteritis was later called Norwalk-like virus (NLV), Norwalk virus/agent, or Small Round Structured Virus (SRSV). While the morphological features of RVs and adenoviruses were obvious, there was confusion about the role of SRSVs found in stool specimens from patients with gastroenteritis symptoms. Within these SRSV/NLV viruses, antigenic differences were observed by IEM (38). Two morphologically different groups were recognized in 1982 (39). SRSVs (known as Norwalk-like or Norwalk viruses) have an amorphous structure with a rough surface, whereas the "classic" calicivirus (Sapporo-like viruses) showed cup-shaped structures from which the calicivirus family was given its name. In the 1990s, genomic information lead to the separation of NLV and Sapporo-like virus (SLV), into two distinct genera within the same *Caliciviridae* family (40). Since then, NoVs were named according the location where each strain was detected. In 2002, NoV became the official genus name approved by the International Committee on

Taxonomy of Viruses (41). However, NoV nomenclature has been challenging, and the lack of an international classification system for NoVs has led to misleading reports concerning certain NoVs. In 2013, Kroneman and co-workers published a standardized nomenclature and classification system, leading to a better understanding of increasing NoV global distribution and epidemiologically important lineages (14).

1.1.2 Norovirus genome structure

The NoV genome is 7.5–7.7 kb in length; it is a single stranded, non-segmented, positive (+) sense RNA virus. The structure of the NoV genome is shown in Figure 1. The genome contains three open reading frames (ORF); the 5' end of the RNA is protein-linked (viral protein genome-linked, VPg) and the 3' end includes a polyadenylated (poly-A) tail. The untranslated region (UTR) at the end of genome is typically short e.g 5' UTR is 2–8 nucleotides (nt) and 3' UTR is typically 48 nt long. (42-44) The UTRs are conserved regions containing RNA secondary structures that are necessary for viral replication, translation and pathogenesis (45). The first ORF is about 5100 nucleotides in size and encodes six nonstructural (NS) proteins. Between ORF1 and ORF2, there is short overlap of 17−20 basepairs (bp) in length, depending on the strain where the NoV recombination occurs; this is also the initiation site for subgenomic RNA transcription (46,47). ORF2 encodes a major structural capsid protein, VP1, and ORF3 encodes a minor structural protein, VP2. Subgenomic RNA contains both VP1 and VP2 proteins. (48)

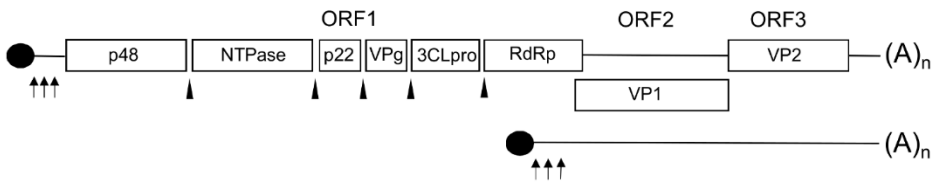

Figure 1. The organization of norovirus genome. Nonstructural proteins in ORF1 are shown in boxes. Protease cleavage sites are marked with arrowheads. Translational initiation codons in genomic and subgenomic RNAs are indicated with arrows. VPgs are shown with black circles. Subgenomic RNA translated from ORF2 and ORF3 is indicated below. Poly-A tails are shown in the end of genomic and subgenomic RNAs. Modified from (48).

1.1.2.1 Nonstructural proteins

ORF1 is synthetized from the N-terminus in the following order: p48, Nucleoside Triphosphate (NTPase), p22, VPg, 3C-like protease (3CLpro), RNA dependent RNA polymerase (RdRp) (Fig. 1). All NS-proteins are involved in the replication of the genome. A functional precursor of NS proteins is not yet clear, but according to the sequence similarity and function of similar proteins of other virus families, certain assumptions can be defined (48). It has been suggested that from the N-terminus, a p48 protein has a role as a scaffolding protein for replication complex assembly. On the other hand, NTPase shows only NTPase activity; it lacks helicase activity. (44,48) The third protein from N-terminal end, protein p22, is present in precursor p22-VPg-3CLpro which are cleaved via proteolytic processing by 3CLpro into separate proteins. At the present, the functions of p22 are not known (49).

Burroughs and Brown described VPg function already in 1978 (50). VPg is covalently attached to the 5' end of genomic and subgenomic RNA. VPg acts as a primer in RNA synthesis in a variety of virus families, including *Caliciviridae*. RNA without a VPg protein is not replication efficiently. The function of the VPg protein is variable; evidence of the function is known from an animal calicivirus study, and the role of VPg in NoV is assumed to be similar in other animal caliciviruses (44,50,51).

The ORF1 encodes the 3CLpro which is called 3C-like protease in analogy with picornavirus 3C. 3CLpro cleaves the polyprotein to the six NS-proteins translated from ORF1. Sequence analysis and site-direct mutagenesis studies have not discovered any additional proteases in human NoVs. (48,52,53) RdRp is required for

genome replication and it is commonly used as a target in diagnostic studies (12). RdRp is required for the initiation of viral RNA synthesis and could be a target for antiviral drug design (53).

1.1.2.2 Structural proteins

Major capsid protein VP1

Major capsid protein VP1 ranges from 520-555 amino acids (aa) and the apparent molecular weight is 58−64 kDa. Prasad and coworkers revealed the NoV structure by X-ray crystallography (54-56). NoV capsid is arranged to $T = 3$ icosahedral symmetry. The capsid has 180 copies of VP1 proteins, or, in other words, the capsid is composed of 90 copies of VP1 capsid dimers. The capsid is 27–32 nm in diameter and does not contain an envelope. (16,56) As presented in Figure 2, VP1 contains the N-terminal region, shell (S) region and protruding (P) regions. The P domain is divided into P1 and P2 subdomains, each corresponding to the leg and the head of the arch-like P-dimer. The amino acid residues that correspond to these domains are indicated in Figure 2. The N-terminal region forms the interior of the virus capsid, S region folds into a classical eight-stranded antiparallel beta-sandwich and forms the continuous surface of the capsid whereas P domain forms the protrusion on the virion. The P2 subdomain is the most exposed one of the capsid structure. Two major domains of the VP1 capsid, the S domain (aa 1−225) and the P domain (aa 225-520) are linked by a flexible hinge. (56)

N-terminal and S regions represent the most conserved region of the capsid protein and is commonly used for diagnostic purposes (57). P1 subdomain is moderately well conserved (56). The P2 domain corresponds to a region of the VP1 that shows the highest sequence variability in NoVs (58). P2 domain has been shown to be an important antigenic site and to contain a receptor binding specificity of the NoVs (59).

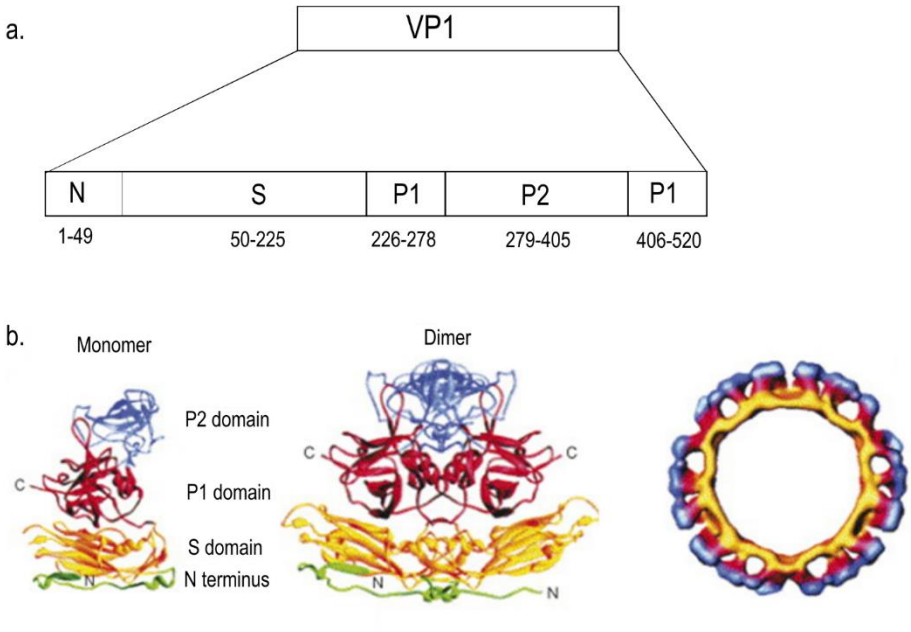

Figure 2. The structure of VP1 genome and major capsid protein. (a) The VP1 genome structure (520 aa) is shown. ORF2 encodes VP1 which contains the shell (S) and the protruding (P) domains. The P domain includes separate P1 and P2 domains. The number of amino acids are indicated under the gene (56). (b) The norovirus capsid structure is defined with colors. N-terminal region is shown in green, the S domain yellow, and the P1 domain in red. The P2 domain is on the top of the capsid surface and is shown in blue. Modified from (60).

Minor capsid protein VP2

ORF3 is approximately 806 nucleotides in size and encodes minor structural protein VP2, which has a molecular weight of 22–29 kDa (61). The VP2 sequence is variable between strains and the NoV virion contains one or two copies of VP2 (61,62). While the function of VP2 is still uncertain, there is evidence that VP2 has a role in RNA genome packaging and function and it is probably involved in the expression and stability of the VP1 major capsid protein (48,63). Recently, it was suggested that VP2 resides in the interior of VP1 and that it may have a role in capsid assembly and genome encapsidation (64).

1.1.3 Norovirus classification

NoV belongs to the *Caliciviridae* family, along with other viruses such as Lagovirus, Nebovirus, Sapovirus and Vesivirus. The viruses of *Caliciviridae* family have been detected in different species such as humans, cattle, pigs, cats, chicken, reptiles, etc. NoV and sapovirus are the two distinct genera that cause gastroenteritis in humans, while other genera represent animal viruses. Feline calicivirus belongs to vesiviruses and cause a respiratory disease in cats and lagoviruses such as rabbit hemorrhagic disease virus causes fatal hemorrhagic disease in rabbits. The neboviruses are the most recently defined to *Caliciviridae* family that cause endemic diarrheal disease in calves. (16,65)

The genus of NoV is further divided into genogroups (GI–VI) (14,66). Genogroup I, II and IV infect humans, causing AGE. GII also infects pigs and GIV infects dogs and lions. GIII has been found in, e.g. sheep and cattle (67,68). Murine NoVs (MuNoVs) belongs to GV and are closely related to human NoVs (69). Recently, GVI has been detected in domestic dogs (15).

Two major genogroups (GI and GII) that infect humans are further divided into different genotypes. GI consists of nine genotypes and GII of 22 genotypes (14). The ancestor of NoVs was NLV, which now designated as GI.1. For the GII genogroup, the similarity of the amino acid sequences in VP1 has to be more than 85% to be within the same genotype (67).

Genotype GII.4 (e.g. genogroup GII, genotype 4) has become the most dominant genotype of NoV causing gastroenteritis outbreaks and sporadic AGE worldwide (70). Genotype GII.4 is further divided into strains or variants (67,71). New pandemic variants emerge almost every two to three years (71,72). Within the same variant, the amino acid sequence of VP1 has been reported to differ up to 2.8% and between the variants the divergence has been at least 5% (73,74).

1.1.4 Genetic variability

RNA viruses evolve by two mechanisms by recombinations and point mutations. Recombination is one of the major driving forces of viral evolution. The recombinant viruses are developed by the ability of viral RNA polymerase to switch the templates while the subgenomic RNA is transcribed by RdRp. The polymerase switches to an available subgenomic RNA from another NoV (co-infection). The result is a recombinant NoV containing ORF1 from one virus followed by new

ORF2 and ORF3 from another virus. The most commonly detected recombination appears close or within the position where ORF1 and ORF2 overlap. (46)

Recombination between different mammalian NoVs is possible, and evidence of zoonotic NoV infections have been reported. Human NoVs can infect gnotobiotic pigs (75) and there is serological evidence of human NoV infections in pigs (76). Antibodies (Abs) against GVI have been detected in veterinarians without evidence that the infections have led to clinical disease (77). The potential of zoonotic infections requires further attention since animals represent a huge reservoir of NoVs where new strains and recombinants may emerge.

Recombination between different NoV genogroups (GI and GII), known as intergenogroup recombination, has been detected (78). In addition, typical recombination hot spots are residing in the ORF2/3 junction as well at the junction of the shell and the protruding domain of ORF2 (described especially in genotypes GII.4). This recombination facilitates the virus to change the elements of genome for antigenic variations and immune evasion, potentially leading to new variants (22).

The high rate of point mutations is typical for NoVs because of the short replication time, lack of proofreading activity of the RdRp, and high multiplicity. Point mutations are mainly reported from the P2 domain of the capsid region of GII.4 variants. Genetic diversity is influenced by environmental selective pressure attempting to maintain a functional RNA genome. (20,23)

1.1.5 Laboratory diagnosis from past to present

There are several methods for NoV detection. Usually, the RT-PCR is used to detect and genetically characterise NoVs.

1.1.5.1 Electron microscopy

In the 1970s, NoVs were detected by EM using a negative staining technique. The disadvantage of the EM was poor sensitivity (minimum of 10^6 particles/ml is required). In addition, the morphology of NoV was challenging to detect since NoVs do not have a calyx surface like sapoviruses do (10,39). Kapikian et al. found the Norwalk agent using IEM in 1972 (10). The virus particles from stool specimens were visualized under EM after reaction with convalescent sera from patients infected with NoV (10). This technique was also applied to characterize other human caliciviruses but the limitation was the lack of specific antisera.

1.1.5.2 Immunoassays

In 1992, the BES for NoVs was developed (27). Briefly, the NoV capsid gene is cloned into the baculovirus (BV) expression vector. The NoV capsid self-assembles to form VLPs in insect cells. This method produces large amounts of VLPs that are morphologically and antigenically similar to the native viruses (79). VLPs were commonly used as antigens in immunoassays to detect Ab responses to infection. In addition, small animals (e.g. mice) were immunized with VLPs to obtain sera for an enzyme-linked immunosorbent assay (ELISA) that could detect NoV antigen in stool specimens. Antigen-detecting ELISA using monoclonal Abs was also developed for the detection of different NoVs in the 1990s (80). These assays are more sensitive compared to the EM methods described above (81).

1.1.5.3 Reverse-transcriptase polymerase chain reaction

The amplification of NoVs by RT-PCR was developed in 1992 (55). The RT-PCR was more specific and sensitive than other methods described above and it became the leading diagnostic and research tool. A variety of RT-PCR methods have been used to detect NVs from stool specimens, water and food (12,82). To increase the sensitivity nested RT-PCRs are used for samples where the virus amount is low like in environmental and food specimens. Real-time RT-PCR detection method is faster than the conventional RT-PCR and with the use of a Taqman probe there is possibility to quantify the virus in a single assay (83,84). Rapid and sensitive multiplex real-time RT-PCR is also used to detect and quantify of NoVs (GI and GII) (85).

1.1.5.4 Genetic analyses

Before DNA sequencing, the positive polymerase chain reaction (PCR) fragments were confirmed and typed to specific genogroups by hybridization. By 1998, DNA sequencing of PCR amplicons allowed the better definition of NoVs (86,87). For investigation of viral taxonomy and to increase the understanding of the clinical significance and epidemiology of the NoVs, sequencing has been a most useful tool. RT-PCR and sequencing together allowed better recognition of the causative agents leading to the finding that NoV was the most common viral agent of foodborne outbreaks worldwide (88).

Many primer pairs have been developed for the detection of genetically diverse NoVs (Fig. 3). The conserved region of the ORF1, namely the RdRp (Region A), is amplified with different primer pairs. In addition, other primers are used to detect the relatively conserved region at the 3' end of ORF1 (region B) and from the beginning of ORF2 (region C and E). For genotyping, the whole capsid gene, VP1 should be analyzed. Vinje et al. showed region D in the capsid gene and demonstrated that the region D and the whole capsid sequences generate identical grouping of strains. (12,19,67) However, the difficulty to amplify region D is resulted in mismatches between primers and template because of the wide variability of NoV genome especially in this part of the genome (89).

For routine detection of NoVs, region C is commonly used because it amplifies the most conserved region of the NoV capsid (the N terminus and the beginning of the S domain). This highly sensitive and specific method allows the detection of NoV capsid genotypes, which is useful especially in cases where a large amount of specimens are to be investigated. In addition, the RT-PCR of region A has commonly been used, which is good for detection of NoVs and polymerase typing but results in the distinct genotype clusters unlike the use of sequences from ORF2. All in all, as recombination is common, both polymerase and capsid genotypes are needed (14,47). In a recent publication, Kroneman et al. suggested that NoV should be genotyped using both polymerase and full capsid VP1 sequences (14).

Sequences are identified by accession numbers, which represent the most related references strains and are unique identifiers given to each sequence when deposited in sequence databases (NCBI Blast® programs and Food-borne viruses in Europe network, FBVE) (90). The sequences are aligned and genetic distances are compared between genotypes in a phylogenetic tree, which is based on the relatedness of nucleotide and amino acids sequences showing the evolutionary relationships between genotypes. The genetic distances between sequences can be calculated and represented in phylogenetic trees. (91)

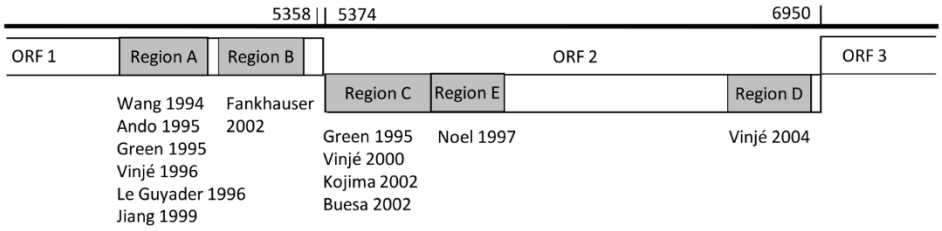

Figure 3. Schematic presentation of norovirus regions used for RT-PCR (A–E) detection and genotyping. The bar represents the genomic location of ORFs in the Norwalk GI.1 virus genome (Accession number: M87661). Modified from (12).

1.2 Norovirus life cycle

Since human NoVs do not replicate in cells cultures, the details of intracellular infection cycle has remained largely unknown. Only recently, a mouse model for human NoVs cultivation has been developed (92). Current knowledge of NoV, attachment, replication and translation is mainly based on findings of MuNoV and feline calicivirus that can be cultivated in cell cultures or studies of NoV VLPs (44,69). MuNoV is widely studied and is used as an alternative surrogate for human NoVs (69,93). The NoV lifecycle is shown in Figure 4.

1.2.1 Host attachment factors and viral entry

HBGAs are carbohydrates of glycoproteins and glycolipids that are expressed on the surface of the mucosal epithelial cells of gastrointestinal, genitourinary, and respiratory tracts. HBGAs are also present on red blood cells, determining the blood group of an individual. (94,95) Saliva and milk also contain HBGAs as free disaccharides (96). The three major HBGAs (ABH, Secretor, Lewis) are regulated by the presence or absence of specific enzymes (fucosyltranferase (FUT) 2, FUT3, A and B) (94). HBGAs act as binding ligands and putative receptors for NoVs. The HBGA binding is located in the P domain of the NoV capsid (97,98). A lack of the FUT2 gene expression makes an individual nonsecretory. About 20% of individuals are nonsecretors and resistant against most but not all NoV genotypes (99-103).

NoVs bind to HBGAs in a strain-dependent manner (29,95,101). In addition, it has been shown that NoVs bind to heparan sulfate proteoglycan and the gangliosides that are expressed on the host cell surface (104,105).

The human NoV entry pathway into the host cells is unknown. MuNoV binds to glycoporteins, glycolipids and sialic acid moieties in a strain-dependent manner and use a clathrin/caveolin-independent mechanism to enter the cell. This mechanism is mediated by dynamin II and cholesterol (106-108). The role of cholesterol has also been shown to have an effect to human NoV infection. Studies with simvastatins, a cholesterol lowering agent, increases NoV replication *in vitro* and *vivo* (109,110). However, the mechanism of this phenomenon is not understood.

1.2.2 Protein translation and replication

When VPg-linked genomic RNA is released into the cytoplasm, the positive-sense RNA acts as messenger RNA (mRNA). Cellular translation initiation factors recognize the viral RNA, which is translated with the cellular translational apparatus. (44) The initiation of translation with VPg is found only in a few animal RNA virus families: *Caliciviridae, Picornaviridae* and *Astroviridae* (51,111,112). However, NoVs exploit the VPg not only for the initiation of translation but also during replication of its genome (51). First, the viral genome is translated to a polyprotein that is cleaved by the virus-encoded 3CLpro.

Major capsid protein VP1 and the minor structural protein VP2 are translated from subgenomic RNA. This strategy enables the production of VP1 capsid proteins in high levels, that are needed for virus assembly, since each capsid contains 180 copies of VP1 (44,58).

The viral genome is replicated by the viral RdRp using both *de novo* and the VPg-dependent mechanism of RNA synthesis (113). RdRp interact with the viral shell domain of VP1 which stimulates the *de novo* inititation (114,115). The viral positive-sense genome serves as a template for negative strand synthesis, which is likewise used as a template for full length genomic RNA and subgenomic RNA transcription (2). Elucidation of mechanism of subgenomic RNA synthesis is based on studies showing both positive- and negative sense forms of the viral genome and subgenome RNA in feline calicivirus-infected cells (114). Thorne et al. showed two models for subgenomic RNA replication (44). The first model suggest that the negative strain synthesis of viral genome undergoes premature termination event. The viral polymerase initiates the replication from the 3' end of the viral genome, but after the

ORF2 is synthetized, a termination signal stops the synthesis. The result is negative sense subgenomic RNA that can serve as a template for positive-sense VPg-dependent subgenomic RNA synthesis. The second model is based on the findings of the highly conserved stem-loop structure in negative-sense RNA downstream of the VP1-coding region. (44) The stem-loop consists of 6 nt and it is common to all *Caliciviridae* family members (45). VPg recognizes the promoter sequence and the polymerase initiates RNA synthesis in the VPg-dependent manner to produce a new subgenomic RNA strand. (44)

1.2.3 Assembly and exit

Mechanism of NoV capsid assembly and exit from the host cell are largely unknown. However, it is known that the major capsid protein VP1 can self-assemble into VLPs *in vitro* that are morphologically and antigenically similar to native virions (27). This suggests that cellular proteins are not necessarily required for the viral assembly process. Minor capsid protein VP2 may have a role in encapsidation but a proper interaction between viral RNA and the VP2 protein has not been demonstrated. The exit strategy of MuNoVs involves the induction of apoptosis, but whether human NoVs use this strategy is unknown. (2,44,116)

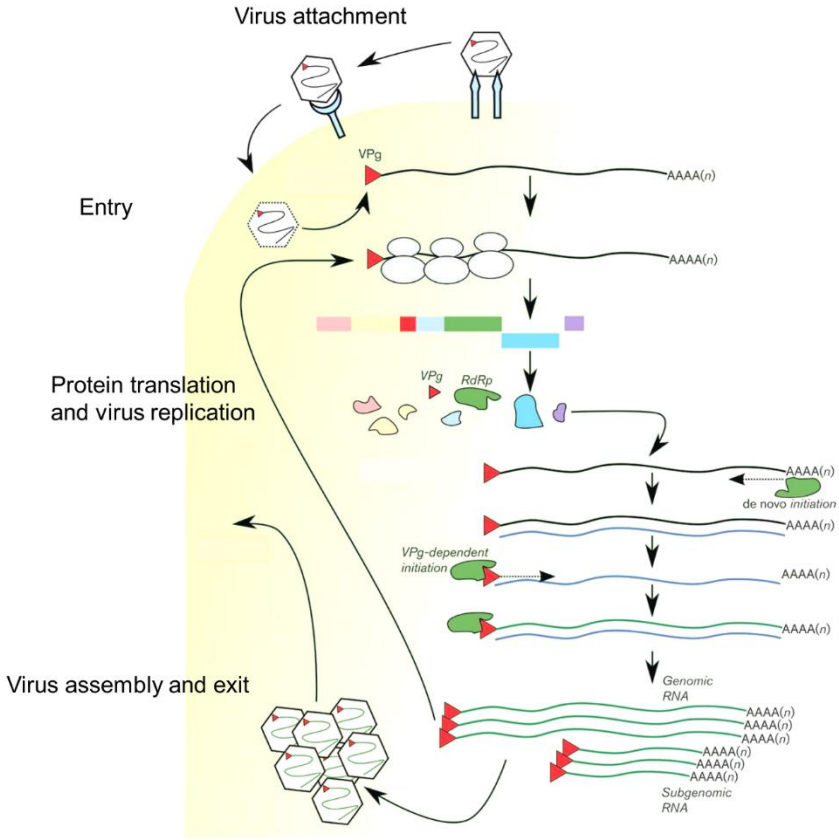

Figure 4. Norovirus life cycle. Noroviruses use various carbohydrate attachment factors on the surface of mucosal epithelial cells. Virus entry and uncoating have so far not well characterized. The viral genome is translated with VPg (represented by red triangle) and cellular mechanisms. The polyprotein is cleaved with proteases, and the viral replication complex is formed. The viral RdRp is responsible for the genome replication from positive strand RNA being used as a template for negative strand synthesis, and vice versa, using both *de novo* and VPg-dependent mechanisms. The replicated genome is packed into viral capsids and then exits the infected cell. Modified from (44).

1.3 Burden of disease and norovirus epidemiology

NoV AGE cases can be divided epidemiologically into four categories: (1) food- and waterborne outbreaks, (2) infections in the immunocompromised and elderly people, (3) sporadic AGE in children and (4) sporadic AGE in adults. The best known and most studied category of these are the outbreaks.

NoV causes approximately 90% of viral outbreaks, 50% of all reported gastroenteritis outbreaks worldwide and up to 58% of gastroenteritis outbreaks in the USA (117,118). NoV outbreaks occur in a wide range of settings, causing high economic costs to affected countries, health-care systems and tour operators (117). Different countries have reported high economic costs because of various NoV outbreaks (119). For example, the United Kingdom's National Health Service has estimated that NoVs cause £-1 billion of economic costs annually (120).

Of the 843 NoV outbreaks reported from 1993 to 2011 in 45 countries, NoVs caused 72,00 illnesses, 501 hospitalizations and 45 deaths. These outbreaks were mainly associated with contaminated food and water transmission (44%) rather than person-to-person transmission of NoVs (16%) and they occurred more often in the communities (66%) than in healthcare settings (26%) (121). NoV was associated with 1908 outbreaks in the USA between 2009 and 2010, causing 69,145 infections, 1093 hospitalizations, and 125 deaths (122). Altogether, severe outcome of NoV infections particularly appeared in healthcare settings, and indeed NoV-associated deaths occurred in the elderly and immunocompromised people. The specific types of settings differ. NoV outbreaks occur more often in long-term care facilities in the US compared to outbreaks reported from Japan, Europe and other high income countries, where NoV outbreaks in acute care hospital are nearly equally common as outbreaks in long-term care facilities (123).

Immunocompromised persons may have a prolonged NoV infection and shed the virus for longer periods than immunocompetent persons (124). NoV-associated deaths have been reported among elderly persons and immunocompromised patient in developed countries, whereas in developing countries NoV-associated deaths occur also in children because of a lack of good healthcare facilities for rehydration (1,124).

Sporadic NoV cases are more frequent among children <5 years of age and adults >65 years of age (125,126). NoV is the leading viral agent after RV to cause clinically significant gastroenteritis among young children, accounting approximately 12% of diarrheal hospitalization worldwide (1). In developed countries where RV vaccination is available, NoV has replaced RV as the predominant cause of viral

gastroenteritis in children. After the introduction of the RV vaccine to the Finnish National Immunization Programme (NIP), RV AGE cases have dramatically decreased. NoV AGE cases, instead, have remained at a constant level. The RV vaccine has been shown not to have any effect on NoV gastroenteritis (3,7). In two pre-NIP seasons (2006–2008) the proportion of NoV-positive AGE cases was 26% whereas in post-NIP seasons (2009–2011) the proportion of NoV-positive cases increased to 34%. (6,7) Similar findings have also been detected in Texas, USA, where NoV prevalence has been almost the same before and after RV vaccination (8). In NoV AGE cases in children <5 years of age, NoV has been estimated to cause >235,000 clinic visits, 91,000 emergency room visits, and 23,000 hospitalizations in the USA, and over 1 million hospitalizations and 200,000 deaths in developing countries (1).

Sporadic NoV AGE in adults is less commonly reported. However, a few studies have described NoV incidence in the community and among outpatients. In Ji'nan, China, NoV was associated with 9% of gastroenteritis cases among outpatients (127). In Beijing, NoV accounted for 12% of cases in outpatients (128). Similar findings were reported in the USA, where NoV incidence rates were 12% in the community and 16% among outpatients (129).

1.3.1 Clinical and epidemiological features of norovirus infection

NoV outbreaks occur year-round, but there is a NoV epidemic peak in the winter months in regions of temperate climate (130,131). Sporadic (non-outbreak) AGE cases caused by NoV in the community occur especially in winter months (November–April) (132-134). Previously, NoV AGE was referred to as a winter vomiting disease.

1.3.1.1 Symptoms

The incubation time of NoV illness is usually 12-48 hours. NoV AGE is associated with acute onset of nausea, vomiting, non-bloody diarrhea, abdominal cramps, and low-grade fever. Symptoms can vary between infected persons. A sufferer may have only diarrhea without vomiting, whereas others may have only vomiting (36,135,136). NoV causes gastroenteritis that is self-limiting, but the illness can also be severe. The duration of illness usually lasts around 1-3 days. Infected persons shed NoV primarily from stools but also from vomit. (132,135,137). The major viral

shedding (approx. 100 billion viral particles/g of feces) primarily occurs after 2-5 days of infection and decreases over time. However, viral shedding may continue even for 4 weeks without any symptoms. (138,139) An infected person can shed NoV even before the onset of symptoms in lower titers and may transmit the virus (140). One third of NoV-infected persons are asymptomatic, but they can still shed the virus in stools even under lower virus loads (126).

Diarrhea

NoV diarrhea is caused by intestinal barrier dysfunction and anion secretion in duodenum. The epithelial barrier dysfunction is related to reduction of sealing tight junctional proteins and an increase in epithelial apoptosis, which may be associated with the lymphocytes which release cytokines and may cause cell deaths (apoptosis). Dysfunction in epithelial cells leads to a leak flux mechanism when water and ions leak back into the intestinal lumen and increase the permeability of the tight junction. Increased anion secretion leads to severe, watery diarrhea. In addition, the reduction of villus surface area paralleled with reduced absorption is associated with NoV diarrhea. (141)

Vomiting

NoV infection causes vomiting that is related to delay in gastric emptying (142). NoV is also believed to cause vomiting by activating the enteric nervous systems similarly to RV (143). NoV activates the vomiting center in the central nervous system that includes the serotonergic afferents (144) and the vagus nerve.

Approximately 50% of children contract NoV infection by the age of two years and 80% of children <5 years of age have contracted NoV infection (145-147). Maternal Abs protect children up to the age of 6 months. Afterwards, children between 7 to 24 months of age are highly susceptible to NoV infection. (146,148-150) Children >1 year of age usually have more vomiting and those <1 year of age have more diarrhea. The median duration of illness decreases when children get older, being the highest (6 days) in children <1 year of age. Vomiting, abdominal cramps, and nausea are more common in children >5 years of age. Children can shed the virus in stools up to three weeks after the onset of illness. (138,151) It has been estimated that children <5 years of age are more contagious than older children and adults (152). Severe diarrhea can lead to dehydration and hospitalization. NoV can also cause severe gastroenteritis in hospitalized children. In terms of severity scores, NoVs cause slightly less severe cases of gastroenteritis than RV (6,132,153,154). Severe symptoms of NoV AGE are particularly associated with NoV GII.4 (6,121,150)

1.3.2 Transmission

NoV is spreading primarily from person-to-person (fecal-oral route) or vomit from an infected person but also from contaminated environments, food, and water. NoV outbreaks typically occur in closed environments (hospitals, day care settings, schools, cruise ships and restaurants) that facilitate person-to-person transmission (123). In addition, food, water, and the environment can become contaminated by an infected person, and another person may ingest the virus after coming into contact with NoV contaminated substances. Typical foodborne NoV illness results from a NoV-infected person contaminating food during preparations and service (155). Food can also be contaminated during food distribution with human waste, e.g. raspberries (irrigated with sewage contaminated water), and oysters growing near the coast (156-158). In addition, NoV transmission via water may result from septic tank leakage or sewage or from breakdowns in chlorination of municipal system (159,160).

There are a number of features that facilitate NoV spread in epidemics, including the low infectious dose of NoV needed, prolonged viral shedding, and high shedding titers. Fewer than twenty viral particles can cause an infection (161,162). In addition, NoVs remain stable over a wide range of temperatures from freezing to 60 °C and persist on different kinds of surfaces and food items, e.g. raw oysters, frozen

raspberries and drinking water. NoVs cannot multiply in food or on surfaces, but they can survive in water and shellfish possibly for months, and they persist on dry surfaces for up to 12 days. NoV is also resistant to common disinfectants (e.g. products containing phenolic compounds, hand sanitizers) (163). Furthermore, the great diversity of NoV strains and high mutation rate has led to a lack of long-term immunity. (1,2,123)

1.3.3 Norovirus genotypes in outbreaks

The common NoV genotypes infecting humans belong to the GI and GII genogroups, of which, GII is responsible for up to 90% of infections (19). Genogroup distribution is associated with transmission routes (164). GI strains are usually the more common cause of waterborne outbreaks as they are more stable in water, and less stable on surfaces (165,166). Between 1983 and 2010, GI strains caused 13% of waterborne outbreaks and multiple strains of GI and GII were associated with 12% of water and foodborne outbreaks worldwide. GII strains alone are typically related to outbreaks in healthcare settings, causing 75% of outbreaks, especially those occurring in winter. These outbreaks have mainly been associated with genotype GII.4. (165,167)

Water- and foodborne outbreaks may occur in both winter and summertime. Here, a few NoV outbreaks are presented to show the high diversity of NoV genotypes in water- and foodborne outbreaks. Polymerase genotype GI.3 dominated as the cause of waterborn outbreaks in Sweden from 2002 to 2006 (165). Between 1998 and 2003, waterborne outbreaks caused by both genogroups GI and GII were noticed in Finland. In these outbreaks, polymerase genotypes GI.3 and GII.4 were the most common genotypes (160,165). A large waterborne outbreak occurred in Nokia, Finland, in 2007. The drinking water was contaminated with sewage and caused AGE symptoms in thousands of people. In this outbreak, the polymerase genotype GII.4 was the most common cause of the NoV AGE cases. (168) In Tampere, Finland, the summer 2014 waterborne outbreak related mainly to the NoV genogroup GI; it involved at least 1093 cases in swimmers (Sirpa Räsänen personal communication).

The capsid genotype GI.3 was the most common genotype in foodborne outbreak in Sweden in 2007 (169). In 2009, an uncommonly large number of foodborne NoV outbreaks was detected in Finland. These outbreaks were associated with contaminated frozen raspberries. The polymerase genotype GII.4 was the most

common finding in the raspberry-linked outbreaks. (170) Both genogroups GI and GII caused foodborne outbreaks that were linked with the consumption of raw oysters in five European countries in 2010 (171). Usually, a high divergence of NoV genotypes are detected in water- and foodborne outbreaks because the contaminated product contains a collection of NoVs circulating in community (163).

1.3.4 Norovirus genotypes in children

Sporadic NoV AGE cases in children (<18 years of age) were primarily caused by a polymerase genotype GII.3 worldwide from 1970 to 1990. Since then, the polymerase genotypes GII.4 and GII.b have dominated. Capsid genotype GII.3 was the most common during the 1990s, leading to the finding that GII.3/GII.3 (polymerase/capsid) was the predominant combination before the 1990s. Over the last decade, the combination GII.b/GII.3 (polymerase/capsid) replaced the previous NoV strain combinations. Nowadays, GII.4/GII.4 (polymerase/capsid) is the most common combination; it cause 54% of sporadic NoV AGE cases in children. (19)

GII.4 caused 89% of NoV AGE cases in children seen at Tampere University Hospital during four seasons (2006–2008, 2009–2011). During the first two seasons, the second most common genotype was GII.b (6%). Other strains were uncommon (GII.7, GII.1, GII.c, GII.2, GI.6). (6,133) Similar findings were seen in the second study period; GII.b caused 14% of NoV AGE cases in children, whereas other strains were seen less frequently (GII.7, GII.g, GI.4, GI.3, GII.e) (7). These NoV genotypes were detected from the polymerase region.

In the last decade, the GII.4 (polymerase/capsid) genotype was observed in 62% and 67% (respectively) of sporadic NoV AGE cases in children. In addition to GII.4, the worldwide circulating polymerase genotypes in children were GII.b, GII.12, GII.7, GII.2 and GII.3, and the capsid genotypes were GII.3, GII.6, GII.2, GII.7, GII.1. (19) Almost all non-GII.4 genotypes were recombinants. The most prevalent recombination, GII.b/GII.3, caused outbreaks at the beginning of 2000, and is still common in children (19).

1.3.5 Epidemiology and specific features of GII.4

Overall, NoV GII.4 has been the most prevalent genotype over the last 20 years among sporadic AGE cases and NoV outbreaks, and it is responsible approximately, for 55-85% of all NoV AGE cases worldwide (70). GII.4 is associated with a greater person-to-person transmission rate and cases of gastroenteritis that are more severe. NoV-associated deaths are more likely caused by GII.4. (73,121,172,173).

Over the last two decades, GII.4 NoVs have circulated and emerged with several variants. The first GII.4 variant was found from the Children's Hospital National Medical Centre in Washington, DC (CHDC-strain) in the 1970s, followed by the Camberwell variant that appeared in the 1980s (73,174). Since 1995, six pandemic variants have emerged: US95/96 1996 (175), Farmington Hills 2002 (120), Hunter 2004 (176), Den Haag 2006b (177), New Orleans 2010 (178), and Sydney 2012 (179). Also other GII.4 variants have circulated and caused only local epidemics in a particular regions: Henry 2001, Japan 2001, Asia 2003, 2006a and Apeldoorn 2008 (22,70,180). It has been shown that both antigenic drift and shift are driving the evolution of the GII.4 genotype. The first four pandemic variants were likely evolved with point mutations in the P2 region of the capsid (antigenic drift), which led to an escape from herd immunity (181,182). Instead, the two latest circulating variants (2010 and 2012) show both antigenic drift with point mutations in the P2 region and antigenic shift with intragenotype recombination at ORF1/2. (23,66).

1.3.5.1 Pre-epidemic forms of GII.4 variants

In novel variants of GII.4, there are eleven recombination hot spots, nine of which are located near the position ORF1/2 interphase. The recent variants are combinations of previously circulating variants that have emerged by recombinations between variants. Variant 2010 is composed of ORF1 derived from variant 2006a and ORF2-3 from variant 2008. Variant 2012 consists of ORF1 from the variant Osaka 2007 and ORF2-3 are related to common ancestor variants 2008 and 2010. These two most recent NoV variants have circulated as pre-epidemic forms causing limited outbreaks in specific regions without a global prevalence. (22)

GII.4 variants have appeared in pre-epidemic forms before acquiring sufficient differences from earlier variants. Pre-epidemic variants 2010 (Orange 2008, GQ845367) and 2012 (Auckland 2010, KF060124) contained similar antigenic epitopes as the ancestors. Pre-epidemic variant 2012 circulated for two years specifically in New Zealand in 2010 (23), and in Canada and Italy in 2011 (183,184).

Before pre-epidemic forms can develop into pandemic variants, several amino acid changes are required in the epitopes. Antigenic variation are essential before the novel variant can escape from herd immunity. (22)

1.3.5.2 Antigenic drift of GII.4 variants

The evolution of the GII.4 lineage is influenced by many factors (172). GII.4 is able to bind to a wider range of HBGAs and have a broader susceptible population compared to other genotypes (185-187). The robust epidemiological fitness of GII.4 has led to an assumption that GII.4 has higher replication and mutation rates than other NoV genotypes (188). The NoV capsid mutation rate has been approximately 10^{-3} nucleotides/substitution/year over the last 34 years (73). These mutations occur mainly in the highly variable P2 domains and these mutations do not reappear. Antigenic variation is localized in major antigenic epitopes, known as blockade epitopes (A–E), in the capsid P2 domain are important factors for the emergence of novel GII.4 variants (71,181). Mutations in these five sites generate antigenically novel GII.4 variants which are able to escape from herd immunity (188,189). The most recent variants, 2010 and 2012, are different in four sites of the P2 domain (aa 294, 368, 373, 376). However, only two sites (aa 294, 368) were critical in determining the specificity of the antigenicity of variant 2012 (21,23). It has been shown that the HBGA-binding profile of GII.4 has changed between variants (173,189,190).

1.4 Immunity

Protective immunity against NoVs in humans is complex and not very well known. The first human challenge study in the 1970s showed that immunity to Norwalk-virus lasts from two months to two years (191). Another challenge study showed that NoV immunity appeared to be only short-term (6–14 weeks) and was lost after two to three years. Homologous Ab protection may last between 8 weeks and 6 months (192). Reinfection with a heterologous strain is possible even within a shorter period. However, in NoV challenge studies, a high dose of virus has been used compared with the natural infection dose of virus. A lower challenge virus dose might lead to the observation of greater immunity and higher level of cross-protection. Recently, Simmons et al. reported that the duration of immunity to NoV lasts four to eight years, based on mathematical modeling (152).

1.4.1 Protection from norovirus infection

Data collected from human challenge studies and natural NoV outbreaks show that people develop an Ab response that does not correlate with protection. Immunoglobulin G (IgG) response persists for months, whereas IgA and IgM responses are detectable only for a short time. (116) The surrogate neutralizing Abs that block the interaction between NoV VLPs and HBGAs are correlated with protection against NoV infection (146,181,193). As discussed earlier, the genetics of the host likely determine susceptibility to NoV infections. Non-secretor individuals (~20% of the population) are less susceptible for NoV infections (101). Secretor-positive individuals have a greater variety of carbohydrates than nonsecretors (29,94,95). A person who is susceptible to infection by specific strains may be resistant to others. It has been shown that high pre-existing genotype-specific Abs in children that block the binding of VLPs to HBGAs are protective Abs, and they prevent NoV infection by the same strain (145,146,194,195). By contrast, high total pre-existing Abs in adults are not protective (192,196) which may be because all Abs are not functional in blocking the virus infectivity with host cell receptor interactions (195).

1.5 Baculovirus expression system generates VLPs

BVs are double-stranded DNA (dsDNA) viruses belonging to the *Baculoviridae* family that naturally infect insects and arthropods (197). They cannot replicate in mammalian or other vertebrates' cells but are found to infect mosquitoes and therefore may transmit to humans (198,199). The BES is a powerful tool in molecular biology and was developed for protein expression during the 1980s. BES was first used to produce human beta interferon in insect cells in 1983 (200). Since then, BES has been used in a wide range of applications for producing foreign proteins in insect cells or silkworm larvae. The advantage of BES is the capability of producing post-translational modifications, because many proteins require folding, subunit assembly, and extensive posttranslational modification to be biologically active (200-202). BES is widely commercialized using the conventional method of homologous recombination *in vitro*. Two vectors are available: *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) and *Bombyx mori* nucleopolyhedrovirus; both contain polyhedrin and polypeptide (p10) promoters. Commonly used insect cell lines are *Spodoptera frugiperda* (*Sf9* and *Sf21*) and *Trichoplusia ni* (under the

commercial name High Five™). The cells optimally grow at 27 ºC without additional CO_2. To date, BES has been used to produce several biologicals components as interferon, antigen and vaccine (95,200,203,204).

1.5.1 Production of VLPs

VLPs have been produced in yeast, plants, bacteria and mammalian and insect cells (205). BES is highly effective in producing large quantities of recombinant viral capsid proteins that spontaneously assemble into VLPs (206). It is feasible to produce both non-enveloped (e.g. NoV, papilloma, RV) VLPs and enveloped (e.g. human immunodeficiency virus, Ebola, influenza) VLPs using BES, although the formation of these VLPs is different in insect cells. (207) Briefly, to produce non-enveloped VLPs, a recombinant BV genome with an insert of capsid protein goes into the nucleus in insect cells. The capsid DNA is transcribed to mRNA, which is exported from the nucleus into the cytoplasm, where mRNA is translated into structural proteins that self-assemble into non-enveloped VLPs. Non-enveloped VLPs are released from host cells during virus-induced cell lysis, causing the breakdown of the cell membrane and cell death (208). By contrast, the production of enveloped VLPs is a more complicated process. The endoplasmic reticulum and Golgi apparatus of infected cells are involved in the formation of the enveloped VLPs (207). VLPs are secreted from the infected cells in a process involving the wrapping of the envelopes around VLPs from the host cells (209,210).

VLPs that are assembled of several capsid gene products (e.g. RV, influenza) can be produced by co-infection or co-expression procedures. Insect cells are transfected with several recombinant BV vectors each containing gene coding for different capsid proteins. Another alternative is that co-expression one polycistronic recombinant BV vector contains several capsid genes and all capsid proteins are produced in insect cells. For the production of RV VLPs, a tricistronic BV vector procedure was a more effective system to produce VLPs than co-infection (211). By contrast, Roldao et al. showed that DNA replication and transcription rates were 50% lower in the co-expression procedures than in co-infection for the production of RV VLPs (212).

The quality of VLPs may vary during the production and purification procedures, and upstream and downstream processing should be adjusted for optimal production of VLPs. (213) To optimize VLP production, agitation, aeration, cell concentration at the time of infection, multiplicity of infection (MOI), and the time

of the infection (TOI) and other parameters should be carefully selected because these factors greatly affect the VLP production level. (207,214)

1.5.2 Purification of VLPs

Purification procedures affect the quality and quantity of a final VLP product (215). Traditional purification methods with sucrose gradient and cesium chloride (CsCl) ultracentrifugation are convenient systems for purification of VLPs (216-219). The gradient ultracentrifugation separates different particles based on their densities (220). The main advantage of these conventional purification methods is the separation of the VLPs from defective virions and cellular debris. The sucrose gradient is created by gently layering a lower concentration of solution on higher concentrations in centrifuge tubes. Finally, the sample is loaded on the top. During the ultracentrifugation the sample, reach the specific density of the gradient, which match with the surrounding solution. The concentrations of sucrose and the speed of centrifugation are depending on the sample. Sucrose/CsCl cushions have also been used for purification. The sample is loaded on the top of the cushion and during ultracentrifugation the sample is pelleted in the bottom of the tube. This procedure does not cause mechanical stress and VLPs remain morphologically intact. However, the purification with CsCl is known to cause aggregation of VLPs and to affect the functionality of VLPs. (215,220)

After conventional purification procedures, the purity of VLPs is usually not greater than 80% (209) because these methods do not differentiate VLPs and BVs (216,221,222). A potential risk for using these VLPs as vaccine candidates is possible contamination with recombinant BVs. For safety and regulatory requirements, a recombinant protein must be free of any infectious virus and genetic material, such as BV DNA. BV is known to have an adjuvant effect in immunological studies (213). It is advisable to remove or inactivate BV in VLP preparations co-produced in insect cells in large amounts (213,223). However, the inactivation process can affect the structure and antigenicity of VLPs. More advanced purification methods are based on chromatography (ion exchange, affinity, gel filtration and hydrophobic interactions). To obtain high yield and pure VLPs a series of chromatographic steps are reuiquired. (215) The ion-exchange is based on the net charge surface of VLPs. Changes in ionic strength and exposure to low pH may cause conformational changes in VLPs (215). Purification methods based on chromatography are being

used to discriminate between VLPs and BVs, resulting in highly pure and good quality VLPs. (224-226)

1.5.3 Features of norovirus VLPs

VLPs resemble native virions; they are not infectious and do not include genetic material (27). VLPs are highly immunogenic compared to purified soluble monomeric proteins (209,227,228). NoV VLPs are stable in wide variety of conditions. NoV VLPs continue to be stable at +4 ºC for at least 6 months and resist -20 ºC. VLPs also remain stable after lyophilization (27). NoV VLPs are stable at pH 3–7, the same pH level as in the gastrointestinal tract, and VLPs persist at temperatures for up to 55 ºC (217).

1.5.4 Norovirus VLPs as immunogens

VLPs resemble authentic virus particles that stimulate humoral and cellular immune system, and they are more efficient than other subunit vaccines. VLPs display the conformational epitopes as native viruses thereby inducing the production of neutralizing Abs and have suitable size for uptake by dendritic cells. (229,230)

NoV VLPs induce both B- and T-cell responses in animal studies (146,219,231). In these studies, both homologous Ab response and cross-reactive Abs between genotypes have been observed. Mice immunized with NoV VLPs produce a balanced level of IgG subtype IgG1 and IgG2a responses that correspond to T helper cell (Th) 2- and Th1-type immune response (232). It has been shown that NoV VLPs produce Abs against conformational and linear epitopes of the NoV capsid (231).

1.5.5 Norovirus GII.4 VLP and antigenic variation

A NoV VLP blocking assay has been used as a surrogate to evaluate the neutralizing activity of both monoclonal Abs and sera (95). The Abs which block the HBGAs and NoV VLP binding are thought to correlate with protection (196). Cross-blocking patterns are highly genogroup-specific (196,233). Antigenic variations, especially in epitopes A, D, and E in the P2 domain of GII.4 are linked to HBGA binding and blocking profiles between VLPs and HBGAs (173).

In summer 2002, a major NoV pandemic emerged that was associated with the novel GII.4 2002 variant (151,234). This variant had a unique insertion, unlike an ancestor variant, in epitope D (aa 393-395). Allen et al. investigated VLP binding to mouse monoclonal Abs between pre- and post-2002 pandemic GII.4 variants. In the Farmington Hills 2002 variant, residues 294 to 296 and 393 to 395 were shown to be important antigenic determinants (235). Molecular characterization of antigenic epitopes together with human and mouse monoclonal Abs have identified and confirmed three epitopes (A, D, and E) between the GII.4 strains that circulated from 1987 to 2009 (173,190,236). Antigenic changes in the P2 domain in genotype GII.4 are associated with changes in potential neutralizing sites (181,190,235,236). Epitope A is highly variable and it often changes with the new emerging strains. Epitope A has been described as being strain-specific, using human convalescent sera and mouse Abs (20). Epitope D has been shown to be involved with HBGA binding affinity (181,190,235), whereas epitope E has been demonstrated to be a neutralizing blockade epitope for variant 2002; in addition, epitope E is strain specific (236). Antigenic changes in these blockade epitopes result in escape from herd immunity (20,190). These changes can affect HBGA binding patterns in a population and determine individual susceptibility to NoV infections. It is assumed that unmapped GII.4 blockade epitopes also exist (173). However, it is unclear whether antigenic variation of GII.4 is permanent. An open question also is whether this could also happen with other NoVs genotypes in appropriate environmental conditions.

1.6 Norovirus vaccines

As discussed previously, NoVs cause approximately 200,000 deaths a year in children under five years of age in developing countries (1). In the USA, 21 million people are infected with NoV every year, causing 70,000 hospitalizations and 800 deaths (237). NoV infections spread easily in places where people are in close contact with each other. NoV causes severe outcomes and deaths in the elderly and immunocompromised patients in healthcare settings (238). The need for a NoV vaccine in these specific groups is obvious, and a NoV vaccine is especially required for other high-risk populations such as children and healthcare workers. In addition, people who are in close contact with the elderly and immunocompromised persons and people who are at high risk for NoV infection such as military personnel, food handlers and travelers should also be vaccinated (239,240).

NoVs do not replicate in cell culture and therefore live attenuated or inactivated vaccines is not possible to produce. A VLP-based vaccine against the hepatitis B virus (Engerix™, GSK and Recombivax HB™, Merck) and the human papillomavirus (Cervarix™, GSK and Gardasil™, Merck) have already been commercialized (241-243).

The most advanced NoV candidate vaccine was the genotype GI.1 VLP developed by LigoCyte. The vaccine contained adjuvants (monophosphoryl lipid A and mucoadherent chitosan) and was adminitered intranasally. In clinical studies homologous B-memory cell response were observed (244,245). In a human challenge study, two doses of intranasal vaccine were given to participants three weeks apart. The participants were challenged with homotypic NoV with ten times the dose required to infect 50% of the recipients. Gastroenteritis and NoV infection were reduced among vaccinated subjects, demonstrating the proof of principle for protection. (246) Another VLP-based NoV bivalent vaccine produced by Takeda Pharmaceutical contains a combination of the genotypes GI.1 and GII.4 consensus strain (2002-2006) with adjuvants (3-O-desacyl-4'-monophosphoryl lipid A and alum). Adult volunteer participants who were enrolled in a clinical trial were secretor positive (functional FUT2 gene) and received two doses of the bivalent vaccine. The bivalent vaccine was well tolerated and was highly immunogenic (30,247). The participants were challenged with the GII.4 variant 2002 which was different from the vaccine strain by 19 amino acids in the hypervariable domain of the NoV capsid. This challenge study showed that a NoV VLP-based vaccine given as an injection can produce cross-protection against severe NoV gastroenteritis but not against NoV infection in adults. (30)

Other NoV candidate vaccines are under development, such as the dry powder NoV-VLP vaccine for intranasal delivery and candidate vaccines based on P-particles (248-250). NoV P-particles induce both humoral and cellular immune responses in mice (251). However, VLPs were shown to be superior to P-particles for inducing cross-reactive B- and T-cell responses (231). P-particles do not present blockade epitopes as efficiently as VLPs. Altogether, it has been suggested that a VLP-based vaccine is a more promising NoV vaccine candidate than one based on P-particles (173).

Neutralizing Ab responses induced by the vaccine are evaluated with the blocking assays and titers in these assays correspond to protection in humans (95,196). Common neutralizing epitopes are not detected between NoV genogroups. Therefore, a NoV vaccine should contain VLPs of both major genogroups even

though LoBue et al. have demonstrate that cross-reactive Abs exist between certain complex combinations of VLPs from GI and GII strains (252).

NoV GII.4 undergoes evolutionary changes via recombinations events and by mutations in the P2 domain. Antigenically distinct novel variants appear every two to three years suggesting that a vaccine may need to be reformulated periodically. Early challenge studies have suggested that protection lasts approximately from 6 months to 2 years, and mathematical modeling based on epidemiological data showed that protection may persist from four to eight years (152,192). NoV vaccine should induce better protection than natural NoV infection, and the vaccine should be multivalent (containing at least one GI- and GII- derived VLP) and capable of inducing long-lasting cross-reactive Abs (31,240).

Our aim has been to produce a combination vaccine against childhood gastroenteritis. The NoV-trivalent vaccine produced in our laboratory consists of GI.3, GII.4 VLPs and RV VP6 protein. All sequences are based on virus strains identified in Finnish patients. VP6 seems to have a natural adjuvant effect that enhances cross-reactive Ab responses against multiple NoVs. Our study group has reported promising results from preclinical studies. The trivalent vaccine candidate induces a strong homologous Ab response and blocking Ab response against other GII.4 variants (2010 and 2012, respectively) and genotype GI.1, suggesting the presence of broadly neutralizing Abs in immune sera. (31,232)

2 Aims of the study

The specific objectives of the study were:

I To determine the prevalence of the norovirus GII.4 genotype and its variants in acute sporadic gastroenteritis in young Finnish children during a 20 year period of 1993 and 2013.

II To compare the clinical features of norovirus infections caused by the predominant genotype GII.4 with those of other norovirus genotypes.

III To produce, purify and characterize norovirus GII.4 VLPs for the vaccine development.

IV To examine the genetic changes of NoV GII.4 variants circulating in Finnish children between 1998 and 2013 in comparison to the GII.4 VLP candidate vaccine strain based on the VP1 sequence from 1999.

3 Materials and methods

3.1 Materials (I-IV)

3.1.1 Clinical specimens from 1993 to 2007

Fecal specimens were originally collected from AGE cases in children up to three years of age who participated in RV vaccine efficacy trials in Finland from 1993 to 2007 and who were prospectively followed for AGE of any severity (253-257). While fecal specimens were collected for RV studies, the same specimens were also available for studies of NoVs. The protocols and informed consent forms were approved by the respective Ethics Committees. AGE was typically defined as at least three or looser than normal stools and/or a vomiting episode in a 24-hour period. Between gastroenteritis symptoms, more than 14 symptom free days had to occur for the cases to be considered as separate episodes. Each study is described in more detail below.

The first RV vaccine trial was conducted in the Pirkanmaa area of Finland; it commenced in September 1993 and ended in June 1995. Stool samples were available from 1586 episodes of AGE in 1200 children. (253) NLV was detected in 313 cases of AGE using a hybridization method (158 in the placebo group and 155 in the RRV-TV vaccine group) (3). The study material from September 1997 to June 1999 consisted of 401 episodes of AGE in Tampere and Lahti (256). The fecal specimens from 1998 to 2001 were collected from AGE cases at four sites in Finland (Espoo, Pori, Tampere, Lahti). From this trial 3630 fecal specimens were available for NoV studies (255). For this study, we randomly selected 1510 samples for NoV genotyping studies. The stool specimens and clinical features (described more closely below) from 2000 to 2002 were collected from infants in five areas (Tampere, Espoo, Lahti, Pori, and Jyväskylä) around Finland; 446 gastroenteritis episodes occurred in 242 infants (254). Fecal specimens from 2001 to 2004 came from the RV Efficacy and Safety Trial (REST) study (257); of these, 825 specimens were selected for our studies. Finally, fecal specimens from 2007 were collected from children who were enrolled in a Rotarix® vaccine (GlaxoSmithKline) trial that commenced in five

European countries, including Finland, in 2004. The infants were followed-up for a third RV epidemic season in 2007 and 106 fecal specimens were available for studies of NoVs. There were no specimens from July 1995 to September 1997 or from June 2004 to January 2007 since there were no vaccine trials going on. For the NoV studies, there was a total of 4727 fecal specimens available from AGE episodes of any severity. All stool samples were stored at -20 °C until tested.

3.1.2 Clinical specimens from 2006 to 2013

More fecal specimens were collected from children under sixteen years of age who were seen at Tampere University Hospital for AGE from September 2006 to August 2008 (6), from September 2009 to August 2011 (7), and from September 2012 to June 2013 (258). The ethics Committee of the Pirkanmaa Hospital District approved all the study protocols. Children with AGE who were treated in the emergency room or admitted to a hospital ward at Tampere University Hospital were enrolled in the studies. Patients were either diagnosed with gastroenteritis (ICD-10 codes: A00-09) or had gastroenteritis symptoms defined as ≥3 loose stool or ≥2 episodes of vomiting or 1 loose stool and 1 episode of vomiting concomitantly with other symptoms. If fewer than seven symptom-free days occurred between the visits to ER or hospitalizations during the study, the AGE symptoms were considered to belong to the same episode. All stool samples were stored at -20 °C until tested.

3.1.3 Clinical features of norovirus GII.4 infection

To study the clinical features of NoV episodes a subset of study patients was analyzed more closely. The information of AGE symptoms was originally collected for an efficacy trial of human monovalent RV vaccine, strain RIX4414 between 2000 and 2002 in Finland (254). If children had gastroenteritis symptoms, the parents kept a daily diary of child's symptoms, reporting rectal temperature, diarrhea and vomiting. A 20-point severity score described by Ruuska and Vesikari was used to assess the clinical features of AGE episodes (153) (Table 1). The score considers the following symptoms and features: fever, duration and intensity of diarrhea and vomiting, and the presence of treatment of dehydration. A score of <7 was defined as a mild, a score of 7–10 as a moderate and a score ≥11 as a severe AGE episode.

Table 1. Numerical scoring system for the severity of norovirus AGE (153).

Symptoms	Score
Duration of diarrhea (days)	
1–4	1
5	2
≥6	3
Max No. of diarrheal stools/24h	
1–3	1
4–5	2
≥6	3
Duration of vomiting (days)	
1	1
2	2
≥3	3
Max No.of vomiting episodes/24h	
0	0
1	1
2–4	2
≥5	3
Fever	
37.1–38.4°C	1
38.5–38.9°C	2
≥39°C	3
Dehydration	
None	0
1–5%	2
≥6%	3
Treatment	
None	0
Polyclinical	
Rehydration	1
Hospitalization	2

3.2 Laboratory methods

3.2.1 RNA extraction (I-IV)

For RNA extraction, a 10% stool suspension was prepared in phosphate-buffered saline (PBS) (pH 7.4). RNAs were extracted from stool specimens using a QIAamp® Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The viral RNAs samples between the years 2000 to 2002 were extracted using a silica binding assay as described previously (259). Aliquots of RNAs were stored at -70 °C until tested by PCR.

3.2.2 Detection of norovirus (I-III)

NoVs were detected from stool specimens using several RT-PCR methods that amplified different regions of the NoV genome with specific primers (Fig. 5, Table 2). The RT-PCR methods used described below.

3.2.2.1 RNA polymerase (region A) (I,II)

The detection of NoVs from stool specimens collected between 1993 and 1995 was done using primer mixture sets (forward primers NI, NVp69 and NVp36, and reverse primer NVp110) (4). These primers detect both sapovirus and NoV-amplifying 397bp, 151bp and 116bp long PCR products from the RdRp region. The RT-PCR methods have been described previously (3).

However, to confirm the NoV findings by the method described above, 297 samples from 1993 to 1995 and the stool specimens taken between 1997 and 2007 were analyzed using the RT-PCR method as published Jiang and co-workers (260) and modification described by Farkas et al. (261). To increase the efficiency of the RT-PCR, the original primers were modified with IUB codes (Table 2). These modified primers were used in primer mixtures with the original primers. This RT-PCR detects both sapoviruses and NoVs from RNA polymerase region A amplifying a 319 bp amplicon for NoVs and a 331 bp amplicon for sapoviruses.

The RT reaction was performed at 42 °C for 60 min with 2.5 µl RNA added to the RT mixture containing 22.9 µl sterile water (Fresenius Kabi AB, Uppsala, Sweden), 1 x GeneAmp® PCR buffer (Applied Biosystems, CA, USA), 1.5 mM

$MgCl_2$ (Applied Biosystems), deoxyribonucleoside triphosphates (dNTPs) 400 μM (Promega, Wisconsin, USA), 16 ng/μl p289,H, I, IUB reverse primer mixture, 10 U RNasin® (Promega) and 70 U M-MLV Reverse Transcriptase RNase H^- enzyme (Promega). A PCR reaction mixture consisting of 26.6 μl sterile water (Fresenius Kabi AB), 2 U GoTaq® DNA polymerase (Promega), 1 x GoTaq® Green Buffer (Promega), 0.5 mM $MgCl_2$ (Promega), and a 24 ng/μl mixture of forward primers (p290, H, I, J, K, IUB) was added to the RT reaction. The PCR was run in a GeneAmp PCR system 9700 or Thermal Cycler 2720 (Applied Biosystems) with the following conditions: primary denaturation at 94 °C for 3 min, followed by 40 cycles at 94 °C for 30 sec (denaturation), at 42 °C for 1 min 30 sec (annealing), at 72 °C for 1 min (extension), and after the cycles' final extension at 72 °C for 10 min. The PCR products were analyzed by agarose gel electrophoresis as described below.

3.2.2.2 Agarose gel electrophoresis

The PCR products were analyzed with agarose gel electrophoresis using a 2% agarose gel with a Generuler™ marker (Fermentas, Vilnius, Lithuania) used as a standard. The amplicons were visualized using EtBr (ethidiumbromide) under UV light with AplhaDigiDoc™ (Aplha Innotec Incorporation, CA, USA). The positive PCR products with the correct size of amplicons were stored at -20 °C for sequencing.

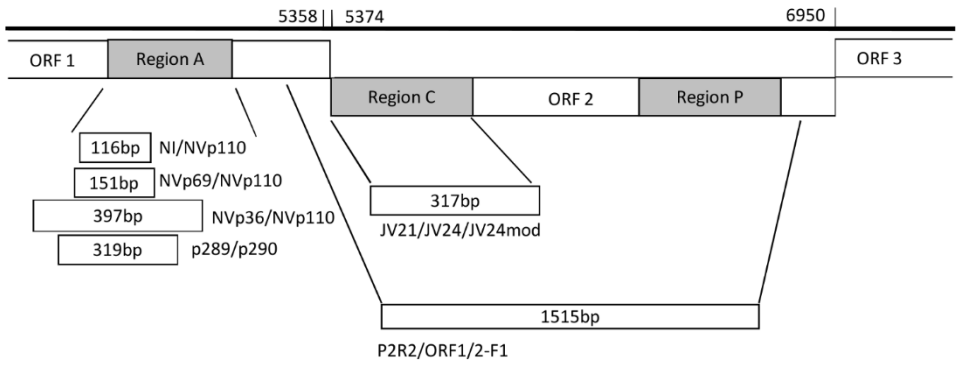

Figure 5. Norovirus genomic regions A, C, and P, which are used for RT-PCRs. The size of PCR products is shown in boxes. Beside the boxes are shown the primers used for RT-PCR. The bar represents the genomic location of ORFs in the Norwalk GI.1 virus genome (Accession number: M87661).

3.2.2.3 Capsid region C (II, III)

To identify NoV capsid genotypes and NoV GII.4 variants, the RT-PCR that amplifies the 317 bp fragment from the beginning of capsid region ORF2 was used (Fig. 5). RNA was reverse-transcribed to complimentary DNA (cDNA) as described by Pang and co-workers (85) with a few changes. Briefly, 2.5 µl RNA was added to the RT-PCR reaction (total volume 20 µl) containing 1 x first strand buffer, 5 µM DTT, 20 U RNaseOut™ recombinant ribonuclease inhibitor, 100 U Superscript™ II reverse transcriptase, 600 ng random primers (all from Invitrogen, Carsbad, CA), and 375 µM dNTPs (Promega). The mixture was incubated at 42 °C for 1 h and inactivated at 70 °C for 15 min. The cDNA was stored at -20 °C.

The PCR mixture (total volume 50 µl) consisting of 5 µl cDNA, 22.5 µl sterile water, 2.5 U GoTaq® DNA polymerase, 1 x GoTaq® Green Buffer, 1 mM $MgCl_2$, 200 µM dNTP (all from Promega), and a 4 ng/µl mixture of each primers (JV21 and JV24) (57) and modified JV24 primer (Table 2). The PCR was run with the same apparatus as described above (Applied Biosystems) with the following conditions: primary denaturation at 94 °C for 3 min, followed by 40 cycles at 94 °C for 30 sec (denaturation), at 49 °C for 1 min 30 sec (annealing), at 72 °C for 1 min (extension), and a final extension after cycles at 72 °C for 10 min. PCR products were run in agarose gel electrophoresis as earlier described.

3.2.2.4 Capsid region P (III)

To define the NoV GII.4 genetic changes in blockade epitopes the primers ORF1/2-F1 (forward) and P2R2 (reverse) were used to amplify the N-terminus and P2-domain of the NoV GII.4 capsid region (235). Extracted RNA was first diluted 1 : 20 in aqua sterilisata (Fresenius Kabi AB). Five microliters of diluted RNA sample was incubated with 1 µl of 25 µM P2R2-reverse primer at 65 °C for 5 min. The RT-reaction mix contained 1 x first strand buffer, 0.5mM dNTP, 0.01M DTT, 40 U RNaseOUT® recombinant ribonuclease inhibitor, and 200 U SuperScript™ III Reverse Transcriptase (all from Invitrogen). Diluted RNA and the P2R2-primer mix was added to the RT-PCR mixture. The RT-PCR reaction was run with the same apparatus as described above (Applied Biosystems) with the following conditions: 25 °C for 5 min, 50 °C for 50 min, and 70 °C for 15min.

The PCR reaction mix contained 0.5 x GoTaq® Flexi Buffer, 1mM $MgCl_2$, 0.2 mM dNTP, 2 U GoTaq® DNA polymerase (all from Promega), and 0.5 µM ORF1/2-F1 forward primer in a volume of 30 µl. The RT-PCR and PCR mixtures were combined together (final total volume 50 µl). The 35-cycle PCR was run using the same apparatus as described above with the following conditions: primary denaturation at 94 °C for 2 min, denaturation at 94 °C for 45 sec, annealing at 58 °C for 45 sec, extension at 72 °C for 1 min 40 sec and a final extension at 72 °C for 10 min. The PCR products were run in agarose gel electrophoresis using a 1% agarose gel as described above.

Table 2. Specification of norovirus primers used for RT-PCR and sequencing

Region	Name	Sense	Sequence (5'−3')	Location of genome
Region A	Nvp110[a]	−	ACGATCTCATCATCACCATA	4864−4884[f]
	Nvp36[a]	+	ATAAAAGTTGGCATGAACA	4486−4505[f]
	Nvp69[a]	+	GGCCTGCCATCTGGATTGCC	4732−4752[f]
	NI[a]	+	GAATTCCATCGCCCACTGGCT	4772−4793[f]
	p289IUB[b]	−	TGACRATKTMATCATCMCCRTA	4865−4887[f]
	p290IUB[b]	+	GATTACTCCARGTGGGAYTCMAC	4568−4591[f]
Region C	JV21[c]	−	CCNRCMYAACCATTRTACAT	5652−5672[f]
	JV24[c]	+	GTGAATGAAGATGGCGTCGA	5355−5376[f]
	JV24mod	+	GTAAATGATGATGGCGTCTAA	5355−5375[f]
Region P	P2R2[d]	−	CCTGCACTCAAACAGAACCCTACC	6530−6554[e]
	ORF1/2-F1[d]	+	CTGAGCACGTGGGAGGGCG	5039−5057[e]
	P2F[d]	+	TGGCAGRTGYACGACTGATGG	5873−5894[e]

[a] (4)

[b] Primers were modified from original primers using IUB codes. Original primers were previously published (260,261)

[c] (57)

[d] (235)

[e] Reference strains NoV GII.4 AY502023. Primer P2F was only used for sequencing.

[f] Reference strain NLV (accession number : M87661)

3.2.2.5 Sequencing and genotyping

All NoV-positive PCR products were sequenced with the same primers used in PCR reactions, except the primer P2F was used only for sequencing the P2 domain (Table 2). Briefly, amplicons were purified with a Qiaquick® Gel Extraction Kit (Qiagen) and sequenced using a Big Dye® Terminator v. 1.1 Cycle Sequencing kit (Applied Biosystems). Sequences were obtained with the ABI PRISM 310 Genetic Analyser. The sequences were analyzed with the Sequencher™ 4.8 software (Gene Codes Corp.,USA). The genotyping and the characterization of NoV GII.4 variants were done using FBVE (www.rivm.nl/mpf/norovirus/typingtool) and GenBank (http://www.ncbi.nlm.nih.gov/Genbank/index.html).

The following accession numbers of NoV genotypes and GII.4 variants were used: GI.2 L07418; GI.3 U04469; GI.4 AB042808; GI.6 AF093797; GII.b at present GII.P21 AY682549; GIIU at present GII.Pj AY682552; GII.d at present GII.P22 AB212306; GII.1 U07611; GII.2 X81879; GII.3 U02030; GII.7 AJ277608; GII.9 AY038599; Camberwell_1994 AF145896; US95/96_1996 AJ004864, AF080551; FarmingtonHills_2002 AY485642; Asia_2003 AB220921; Kaiso_2003 AB303929; Hunter_2004 AY883096; Terneuzen_2006a EF126964; DenHaag_2006b EF126965, OC07138/2007/JP_AB434770; Apeldoorn_2008 AB445395; NewOrleans_2010 GU445325; Sydney_2012 JX459908. The accession numbers and the GII.4 variants' names from FBVE were used for NoV GII.4 sequence nomenclature. However, all findings in this study were from the original strains extracted from the AGE children, not from the reference strains.

3.2.3 Statistical analyses (II)

The comparisons of clinical symptoms (duration of diarrhea and vomiting (days), maximum number of diarrheal stools and vomiting episodes in 24 h, and age in months) between NoV GII.4 and other NoV genotypes were conducted with a two-tailed Mann-Whitney U-test using IBM SPSS Statistics-software version 15.0. Using the following data set, the medians of the severity score points were calculated and analyzed using the same method of analysis as described above. Results were considered to be statistically significant at $p<0.05$.

3.2.4 Production of norovirus VLPs (IV)

3.2.4.1 Cloning of the norovirus GII.4 capsid protein gene

The NoV GII.4 (the closest reference strain: AF080551) fecal specimen was extracted from a patient stool sample collected in 1999 in Finland. The VP1 capsid gene sequence (1.6kb) was amplified with the following primers: forward (5'-GTGAATGAAGATGGCGTCGA-3') (57) and reverse (5'-TTATAATGCACGTCTACGCCC-3') and further amplified with the same primers including restriction sites that were required for the cloning step. Next, the VP1 capsid sequence was cloned to the pCR 2.1 TOPO-vector and subcloned to pFastBac1 and transformed to the competent *Escherichia coli* cells. The cloning steps of the NoV GII.4 full length capsid genehave been described in more detail elsewhere (262).

3.2.4.2 Norovirus capsid expression and VLP production

To produce VLPs, the Sf9 insect cells (Invitrogen) were infected with the recombinant baculovirus. In more detail, Sf9 cells were seeded in Multidish 6-wells (Nunc, Thermo Fisher, Scientific, Roskilde, Denmark) at 1 x 10^6 cells/ml of serum-free medium (Sf 900 SFM III; Invitrogen) and transfected by bacmid DNA with insert (1 μg) using Cellfectin (Invitrogen). The cells were grown at 26 °C and harvested 72 h post-transfection. The cell suspension was centrifuged at 500 x g for 5 min and the supernatant (P1 baculovirus stock) was aliquoted and stored at 4 °C. Sf9 cells were infected with the baculoviral P1 stock and after six days post-infection (dpi), the cell suspension was centrifuged for 5 min at 500 x g and the aliquoted supernatant (P2 baculovirus stock) was stored at 4 °C.

A BacPak Rapid Titer kit (Clontech Laboratories, Mountain View, CA) was used to determine the baculovirus titer as the multiplicity of infection (MOI) of the P2 stocks according to manufacturer's instructions. The application of the kit is described more closely later in this dissertation (see section 3.2.8.2). For the production of VLPs, 200 ml Sf9 cell cultures were set up at a density of 1 x 10^6 cells/ml and cells were infected with P2 stock at a MOI of 0.1, 1 or 5. Aliquots of the cell suspension were collected daily for up to 6 days and analyzed by SDS-PAGE for capsid protein content. The supernatants were separated from the cells by centrifugation at 3000 x g for 30 min at 4 °C. The cells were resuspended in PBS and

adjusted to a volume equal to that of the supernatants. Equal amounts of the supernatant and the cell lysate were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

3.2.5 Density gradient purification procedures of norovirus VLPs (IV)

3.2.5.1 Sucrose density gradient purification

VLPs were isolated from the infected cell culture (200 ml) at day six by centrifugation at 3000 x g for 30 min at 4 °C and purified with several procedures (Fig. 6). The supernatant was ultracentrifuged (L8-60M ultracentrifuge, Beckman SW-32.1 Ti rotor) at 100,000 x g for 2 h at 4 °C and VLP pellets were resuspended in 3 ml 0.2 M Tris-HCl, pH 7.3. The VLPs were loaded onto a 10%–60% discontinuous sucrose gradient and ultracentrifuged at 100,000 x g for 1 h at 4 °C as described before (58,263). Fractions were collected by bottom puncture, the aliquots were analyzed by SDS-PAGE, and the fractions containing VLPs were pooled. To further purify the VLPs, an additional discontinuous sucrose gradient (35%–60%) was accomplished by ultracentrifugation at 100,000 x g for 1h at 4 °C. Fractions containing VLPs were collected and pooled. Sucrose was removed by overnight dialysis against 1 l PBS. VLPs were concentrated by dialysis against polyethylene glycol (PEG; 50%) (264) or by ultrafiltration (265). Briefly, up to 15 ml of dialyzed product was concentrated using an Amicon Ultra 30 kDa centrifuge filter device (Millipore Corporation, Billerica, Germany) and centrifuged using a swinging bucket rotor at 4000 x g for 30 min. VLPs were stored at 4 °C in PBS.

Alternatively, the supernatants were ultracentrifuged twice at 100,000 x g for 2 h at 4 °C, the pellets were resuspended in 0.2 M Tris-HCl, pH 7.3 and placed on a discontinuous sucrose density gradient (10%–60%) for centrifugation at 100,000 x g for 16h at 4 °C. A visible VLP band at the 35% sucrose layer was collected (266). Sucrose was removed by dialysis against 1 l PBS and VLPs were concentrated by ultracentrifugation at 100,000 x g for 2 h at 4 °C and resuspended in PBS.

3.2.5.2 Cesium chloride purification

Clarified supernatants were concentrated as described above for the sucrose gradient purification. The pellets were resuspended in 3 ml sterile water (Aqua sterilisata,

Fresenius Kabi) and sedimented through CsCl (0.4g/ml) by ultracentrifugation at 116,000 x g for 18 h at 4 °C (Beckman SW-55 Ti rotor) as described earlier by others (217,219). Fractions (500 µl) were collected from the top of the ultracentrifuge tube and the aliquots were analyzed by SDS-PAGE. Fractions containing VLPs were pooled. The CsCl was removed by dialysis against PBS and the VLPs were concentrated with an Amicon Ultrafilter device as described above.

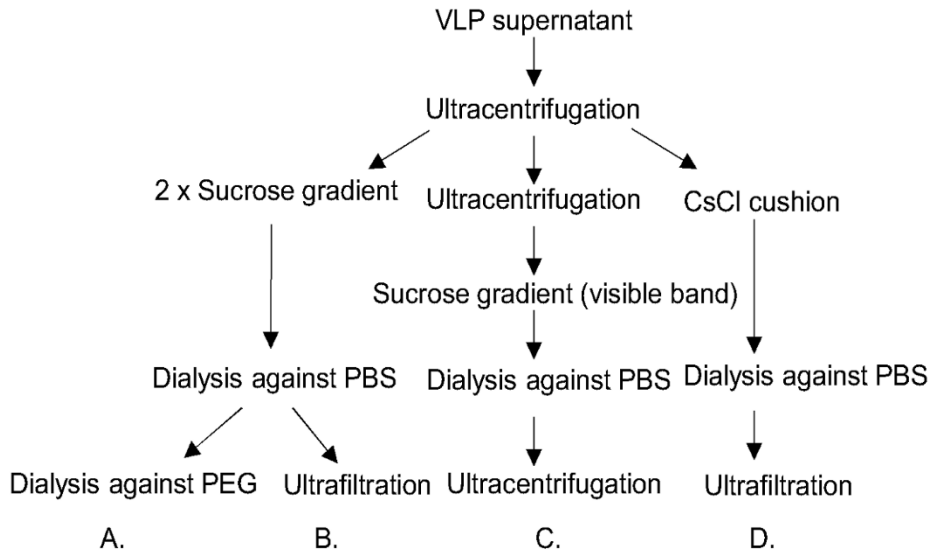

Figure 6. Purification and concentration schemes of norovirus VLPs. Several purification procedures were compared (A–D) considering purity, morphology, antigenicity and yield.

3.2.6 Characterization of expression of norovirus VLPs (IV–V)

3.2.6.1 SDS-PAGE

NoV capsid protein expression was confirmed by SDS-PAGE. Analysis of proteins by SDS-PAGE was done using polyacrylamide gels with 12% acrylamide in the separating gel and 5% in the stacking gel (Biorad Laboratories, Hercules, CA).

Samples were boiled for 5 min in Laemmli sample buffer containing 2% SDS, 5% β-mercaptoethanol, 62,5 mM Tris-HCl (pH 6.8), 25% glycerol and 0.01% Bromophenol Blue (Biorad Laboratories). Gels were stained with PageBlue™ Protein Staining Solution (Fermentas).

3.2.6.2 Protein content and endotoxin level

The total protein contents of the preparations were quantified by the Pierce BCA Protein assay (Thermo Science, Rockford, USA) according to the manufacturer's instructions, with bovine serum albumin (BSA) used as a standard. The optical density (OD) at 544 nm was determined with a Victor2 1420 Multilabel Counter (Wallac Oy, Turku, Finland). The limulus Amebocyte Lysate (LAL) assay (Lonza, Walkersville, MD, USA) was used to quantify the endotoxin levels in the purified VLP preparations. The level was <0.1 EU/10 μg of protein; below the international standard of <30 EU/20 μg protein (267).

3.2.6.3 Immunoblotting

The NoV capsid proteins were first run in SDS-PAGE gel and then transferred onto a nitrocellulose membrane (Trans-Blot, Transfer membranes, Biorad). The capsid proteins were detected using convalescent serum of an individual known to be infected with NoV GII.4 by RT-PCR detection from a patient stool sample. The assay dilution was 1:500 in 1% milk and 0.05% Tween 20 in Tris-buffered Saline (TBS). Horseradish peroxidase (HRP) goat anti-human IgG (Invitrogen) was used as a secondary antibody at a dilution 1:10,000 in 1% milk, 0.05% Tween 20 in TBS. The membranes were developed colorimetrically using the an Opti-4CN detection kit (Biorad) according to the manufacturer's instructions.

3.2.6.4 Electron microscopy

The VLP preparations were negatively-stained with 3% uranyl acetate (UA) (pH 4.6). Three μl of the VLP sample was applied to a carbon-coated grid for 30 sec. The grid was dried with filter paper and 3 μl of UA was applied to the grid for 30 sec. Excess liquid was removed and the grid was examined with a FEI Tecnai F12 electron

microscope (Philips Electron Optics, Holland) operating at 120 kV. NoV VLPs were obtained at magnification of x 30,000 and a bar of 100 nm was used.

3.2.6.5 HBGA binding assay (IV)

The binding of GII.4 VLPs to synthetic carbohydrate receptors was examined by the HBGA binding assay as described previously (193) with slight modifications. Briefly, high-binding enzyme immunoassay plates (Nunc Maxisorp, Thermo Fisher Scientific) were coated with VLPs at 50 ng/well in PBS. Plates were incubated for 4 h at room temperature (RT), washed five times with PBS-Tween 20 (0.05%) and blocked overnight at 4 °C with 5% milk in PBS. After washing, synthetic biotinylated H type 3 histo-blood group carbohydrates (Lectinity Holdings Inc. Moscow, Russia) were diluted to a 6 μg/ml concentration in 1% milk, Tween20 (0.05%) in PBS, and serially diluted threefold in the microtiter duplicate wells. The plates were incubated for 4 h at 37 °C. After the final washing step, HRP-conjugated streptavidin (Pierce, Thermo Fisher Scientific) was added (1:2000) and the plates were incubated for 1 h at 37 °C and washed as previously. After the final washing step, the bound HRP-conjugated streptavidin was detected with SigmaFAST O-Phenylenediamide Dihydrochloride (OPD) at 0.4 mg/ml (100 μl/well) (Sigma Chemical Co., MO, USA). ODs were measured at 490 nm in the Victor2 1420 Multilabel Counter (Wallac Oy). Wells lacking the synthetic carbohydrates were used as a negative control.

3.2.7 Purification of VLPs by ion-exchange chromatography (V)

To obtain pure NoV GII.4 VLPs chromatographic purification was applied using a column with anion exchangers according to the manufacturer's instructions (5 ml HiTrapQ, GE Healthcare, Uppsala, Sweden). Briefly, to remove preservatives, the column was first washed with a start buffer consisting of 50 mM sodium phosphate (pH 6.6) and then with an elution buffer consisting of 0.5 M NaCl in 50 mM sodium phosphate (pH 6.6) and equilibrated with the start buffer. Density gradient-purified GII.4 VLPs were dialyzed against the start buffer and then 500 μg NoV capsid protein VLPs were loaded into the column using a syringe. The column was washed with the start buffer to remove of unbound VLPs. Next, the elution buffer was added to the column and unbound proteins were issued to 26 fractions (500 μl/each). The fractions were analyzed by SDS-PAGE and the fractions with the

NoV capsid were pooled and dialyzed against start buffer to be ready for additional chromatographic purification as described above. The fractions free of BV glycoprotein (gp) 64 according to SDS-PAGE and immunoblotting were pooled, dialyzed against PBS, and sterile filtered using a 0.2-µm syringe filter (VWR, Darmstadt, Germany).

3.2.7.1 Production and purification of mock baculovirus preparation

BVs were prepared using a baculovirus expression kit, namely the Bac-to-Bac® Baculovirus expression system. However, the pFastBac plasmid lacking the insert was transformed to the competent DH10Bac Escherichia coli cells and the bacmid DNA was isolated with a PureLink® HiPure Plasmid Miniprep kit (Invitrogen). The transfection of Sf9 cells with bacmid DNA and P1 and P2 stocks was done with similar methods as described earlier the section detailing the production of NoV VLPs. P2 BV stock was used to infect 200 ml Sf9 cells at 1 x 10^6 cells/ml at MOI of 1. The same apparatus as described in the production of NoV VLPs was used for the production of the BV preparation. The cell culture was clarified at 6 dpi by centrifugation at 3000 x g for 20 min at 4 °C. BVs were pelleted in the suspension by ultracentrifugation at 100,000 x g for 1.5 h at 4 °C. The pellets were resuspended in 0.2 M Tris-HCl, pH 7.3. The BV pellets were loaded on the top of a 10%–60% discontinuous sucrose gradient and ultracentrifuged at 100,00 x g for 3 h at 4 °C. Fractions were collected and analyzed by SDS-PAGE as described above. The fractions containing BVs were pooled and dialyzed against PBS, pH 7.4. The sample was concentrated using the Amicon Ultra 50 kDa centrifuge filter device (Millipore Corporation) and stored in PBS at 4 °C.

The mock BV preparation was further purified with anion exchange chromatography using similar methods as described above in the NoV VLP section. The fractions from chromatographic column (26 fractions, 500 µl each) were collected and analyzed by SDS-PAGE.

3.2.8 Detection of impurities (V)

3.2.8.1 Determination of the proteins

Density gradient-purified NoV VLPs and BV preparations were analyzed by SDS-PAGE. The fractions from the anion exchange chromatography were analyzed by SDS-PAGE (as described above) and stained with PageSilver™ Silver Staining Solution (Fermentas) according to the manufacturer's instructions to result in higher sensitivity of protein detection. The protein purity of the NoV VLPs was determined by densitometric analysis from Page Blue-stained SDS-PAGE gels using AlphaEasy® FC Software (Alpha Innotech, San Leandro, CA). In this analysis, the controls were Sf9 cell lysate and mock BV preparation.

The antigenicity of the proteins was detected by immunoblotting. The method is described above, with only the primary and secondary antibodies being different. NoV GII.4 was detected with the NoV GII.4 monoclonal antibody (Kim Laboratories Inc., Illinois, USA) at a 1:4000 dilution, whereas BV gp64 was detected with the anti-gp64 monoclonal antibody at a 1:400 dilution. HRP-conjugated goat anti-mouse IgG (Sigma-Aldrich) was used as a secondary antibody to detect the NoV capsid protein at a 1:10,000 dilution, and at a dilution 1:1000 to detect BV gp64.

The morphology of NoV VLPs and BV were detected by EM as described above.

3.2.8.2 Determination baculovirus titer

A BacPAK™ Rapid Titer Kit (Clontech) was used to determine the live BV titer according to the manufacturer's instructions. Early log-phase Sf9 cells (6.5 x 10^4 cells/well) were seeded on a 96-well microtiter plate (Nunc™) in Sf-900 cell medium. The plates were incubated for 1 h at 27 °C and the medium was aspirated from the wells. Then the cells were infected with serially diluted samples and incubated for 1 h at RT. After the samples were aspirated from the wells the cells were covered with methyl cellulose. The cells were incubated for 43−47 h at 27 °C and fixed with 4% paraformaldehyde (Sigma Aldrich). The live BVs were detected with the monoclonal gp64 antibody and HRP-conjugate provided in the kit. The foci of infections were counted in duplicate wells with an inversion microscope (Nikon, Badhoevedorp, Netherlands). The plaque-forming units (pfu/ml) were calculated. The average number of foci per well was multiplied by the corresponding dilution factors and an inoculum volume normalization factor of 40.

3.2.8.3 Analysis of baculovirus genome

BV DNA was quantified from the chromatographically purified NoV VLP preparations using a BacPAK™ qPCR Titration Kit (Clontech) according to the manufacturer's instructions. Briefly, BV DNA was purified from the preparations using the NucleaSpin® Virus Kit included in the BacPAK™ qPCR Titration Kit. Pure NoV VLPs, density gradient-purified VLPs (positive control) and Sf9 cell lysate (negative control) were serially diluted from 10^{0} to 10^{-2}. The Kit included the AcMNPV vector that was used as a standard (detection range from 1.4 x 10^{0} to 1.4 x 10^{8} copies/µl). The detection of the BV DNA genome content was based on SYBR® Green technology. The PCR reactions were run in a 7900HT Fast Real-Time PCR machine (Applied Biosystems) using a 96-plate format with the following conditions: primary denaturation at 95 °C for 30 s, denaturation at 95 °C for 3 s, annealing/extension at 60 °C for 30 s. The sample's, copy-number was back-calculated considering the DNA extraction and the dilution of the samples.

3.2.8.4 Quantification of total DNA content

The total DNA was quantified using a Quant-it dsDNA Broad-Range Assay Kit (Invitrogen) according to the manufacturer's instructions. Lambda phage DNA was used as a standard. Fluorescence at excitation/emission 485/535 nm was determined by the Victor2 1420 Multilabel Counter (Perkin Elmer).

3.2.8.5 Sterility test

For the detection of possible contamination factors in the NoV VLP preparations, two culture media were used. Fluid thioglycate and soybean-casein digest medium (both from Sigma Aldrich) were recommended for sterility testing by the World Health Organization in 2012 (268). Both tests were utilized according to the manufacturer's instructions.

3.2.9 Immunogenicity (V)

3.2.9.1 Animals

Female BalB/c mice (4 mice/group, at the age of 7 weeks) were immunized with NoV VLPs using two doses, 1 μg and 10 μg intradermally (ID) at weeks 0 and 3. Blood samples were taken in study weeks 0, 2, 3, and 4. Mice were euthanized at 2 weeks after the second immunization and whole blood was collected. Unvaccinated mice were used as a control. The protocol for animal study was approved by the Finnish National Animal Experiment Board (permission number ESLH-2009-06698/Ym-23).

3.2.9.2 ELISA assays

Immunogenicity of the purified NoV VLPs was determined by ELISA from mice sera specifically for NoV GII.4 IgG, IgG1, and IgG2a (231,232). The IgG response was analyzed from individual mice sera in study weeks 0, 2, 3, and 4, whereas the IgG1 and IgG2a immune responses were detected from pooled sera. Briefly, at 0.2 μg/ml (100 μl/well) GII.4 VLPs were coated on the wells. Serum samples were diluted at 1:200 or twofold dilution series were used. HRP-conjugated anti-mouse IgG (Sigma-Aldrich) was utilized at a 1:4000 dilution whereas the IgG subtypes were determined using the IgG at a 1:6000 dilution.

3.2.10 Alignment and phylogenetic analyses of the GII.4 strains (III)

To compare the major antigenic epitopes of the eight most common GII.4 variants that circulated in Finland between 1998 to 2013 and the GII.4 VLP vaccine, the amino acids sequences of the P2 domains were aligned using Clustal X (version 1.8) and GeneDoc (version 2.7.000). The phylogenetic tree was generated using the neighbor-joining method by comparing the P2 amino acid sequences (aa 142) obtained for nine NoV strains using MEGA 3.1 software (269) with a bootstrap value 1000. Genetic distances were calculated using the Poisson correction method.

4 Results

4.1 The prevalence of norovirus infections in Finnish children (I, III)

Fecal specimens from AGE cases of children under three years of age who participated in various RV vaccine trials between 1993 to 2007 were analyzed. Previous studies had shown that a RV vaccination did not affect the prevalence or severity of NoV AGE (3,270). Therefore, specimens from placebo and vaccine recipients were combined for the NoV studies. A total of 4727 fecal specimens were studied and 1172 (25%) episodes were found to be associated with NoVs. Most NoV AGE cases occurred in wintertime. Usually, the annual NoV epidemic season was from November to April. The NoV season was defined as starting in July and ending in June next year.

4.1.1 The distribution of genotypes in Finnish children

According to the polymerase region A, thirteen genotypes were detected among the 1149 cases of NoV AGE in Finnish children from 1993 to 2007. Table 3 shows the annual distribution of the five most common NoV genotypes belonging to genogroup II. Altogether, GII was accounted for 96% of all NoV AGE cases in Finnish children. The distribution of the NoV genotypes was most diverse in the seasons 1999–2002.

NoV GII.4 was the most common genotype being responsible for 46% of all NoV AGE cases throughout the study period. In the earliest study years, GII.4 was circulating mainly with GII.3 and GII.7 (1993–1998). However, NoV GII.4 dominated in 1998−1999, 2002–2003, and 2006–2007, which were identified as the peak years. The prevalence of NoV GII.4 was the lowest in season 1999–2000 (13%) and in season 2003–2004 (17%) when genotypes, GII.7, GII.3, GII.1 and GII.b, dominated (Table 3).

The second most common genotype, GII.7 (15%) circulated throughout eight seasons but never dominated. Instead, the third most common genotype, GII.3

(14%), dominated seasons 1993−1995. The fourth most common genotype, GII.1, occurred in four seasons from 1998–2002. Genotype GII.b (7%) was the predominant genotype in season 2003–2004 after the peak year 2002–2003 caused by the genotype GII.4. Other genotypes appeared rarely. Genogroup I was detected in seasons 1994–95 and 1998–2002 but it was responsible for only a few NoV AGE cases in children.

Table 3. Norovirus genotypes in sporadic cases of acute gastroenteritis in Finnish children from 1993 to 2007.

Genotype	Season[a]										Total (%)
	1993–1994	1994–1995	1997–1998	1998–1999[b]	1999–2000[b]	2000–2001[b]	2001–2002	2002–2003	2003–2004	2006–2007	
GII.4	6	113	27	148	24	48	57	45	3	56	527 (46%)
GII.7	3	13	13	29	57	34	18	3			170 (15%)
GII.3	6	141	10	4	5	1					167 (14%)
GII.1				7	93	4	2			1	107 (9%)
GII.b					3	15	43	2	15		78 (7%)
GI[c]		2		4	9	14	10	1			40 (4%)
Other[d]		10	8	4	6	9	23				60 (5%)
N[e]	15	279	58	196	197	125	153	51	18	57	1149

[a] Season is the time period from July to June.
[b] 438 (29%) cases of NoV positive specimens were randomly selected for NoV genotyping.
[c] GI genotypes: GI.3, GI.6, GI.4, GI.2
[d] Other genotypes detected (GII.2, GII.9, GIIU, GII.d).
[e] Total number of NoV positive specimens

4.1.2 Prevalence of GII.4 between 1998 and 2013

To further confirm the genotype and variants of the GII.4 cases in the community in years the 1998–2007, all NoV GII.4 specimens were also examined according to capsid region C. In addition, 424 NoV-positive specimens collected from children seen at Tampere University Hospital because of AGE from August 2006 to June 2013 were used. NoV genotypes from hospital-based study (2006−2013) materials that had been studied for polymerase region A (6,7,258) were studied for capsid

region C. Of these, NoV GII.4 variants have been described earlier in one season, 2009–2010, in Finland (271).

We combined the study materials of sporadic NoV AGE cases in the community and hospital-based NoV AGE cases (Table 4). Altogether, NoV was detected in 1495 non-outbreak AGE cases in children between the years 1998 and 2013. Of those, 829 (55%) were caused by the NoV GII.4 genotype. In children with NoV AGE seen at Tampere University Hospital GII.4 was found in 79% of the cases (Table 4).

4.1.3 Norovirus GII.4 variants

In twelve NoV seasons, GII.4 was the predominant genotype with new variants emerging almost every second year (Table 4). In the season 1998–1999, variant 1996 was the only GII.4 variant seen. It circulated in Finnish children from 1998 to 2002. Variant 2002 emerged during the season 2000–2001 and circulated from 2000 to 2007, causing an epidemic peak in 2002−2003. Surprisingly, two GII.4 variants, 2006a and 2006b, emerged in the same season, 2006–2007, together causing the most severe NoV epidemic season in children. Variant 2006b predominated in hospital-based NoV AGE cases, causing almost four times as many NoV AGE cases as variant 2006a. Variant 2008 was detected only in a few hospital-based NoV AGE cases during season 2009–2010. A new variant, 2010, was first detected in season 2009–2010 but it continued to be found from 2009 to 2013. In the last study season, 2012−2013, variant 2012 emerged, being responsible for 63% of all NoV GII.4 AGE cases. Altogether, we found six GII.4 variants (1996, 2002, 2006a, 2006b, 2010, 2012) that were associated with most of the NoV AGE cases in children. The two most common variants were the 2006b and 1996 (Table 4).

Table 4. Norovirus GII.4 variants circulating in Finnish children from 1998 to 2013.

	Season[a]												Total
	1998–1999	1999–2000	2000–2001	2001–2002	2002–2003	2003–2004	2006–2007	2006–2007	2007–2008	2009–2010	2010–2011	2012–2013	
NoV GII.4	213 (72%)[b]	57 (19%)[c]	68 (37%)[d]	49 (32%)	48 (86%)	3 (17%)	56 (98%)	138 (95%)	78 (76%)	37 (74%)	36 (59%)	46 (71%)	829 (55%)
Camberwell_1994		1											1
US95/96_1996	76	28	23	20	9								156
Farmington Hills_2002			1	19	39	1	1						61
Asia_2003							1						1
Kaiso_2003			1	10									11
Hunter_2004						2		5					7
Terneuzen_2006a							26	46	1				73
DenHaag_2006b							27	87	77			8	199
OC07138_2007							1			1			2
Apeldoorn_2008										3			3
New Orleans_2010										33	36	9	78
Sydney_2012												29	29
N[e]	294	308	186	152	56	18	57	146	102	50	61	65	1495

[a]Season is the time period from July to June. [b]76 (36%) cases of NoV GII.4 positive specimens were randomly selected for GII.4 variant identification. [c]29 (51%) cases of NoV GII.4 positive specimens were randomly selected for GII.4 variant identification. [d]25 (37%) cases of NoV GII.4 positive specimens were randomly selected for GII.4 variant identification. [e]Total number of NoV positive specimens. The line separates the community-based and hospital-based studies.

4.2 Norovirus GII.4 clinical features (II)

NoV GII.4 genotype was the most common genotype among all NoV AGE cases in Finnish children and it predominated in hospital-based NoV AGE cases. To assess whether the NoV genotype GII.4 was more virulent than other genotypes, the clinical features of NoV GII.4 infections were studied. Since pre-existing immunity from old NoV infections may ameliorate the clinical course of NoV gastroenteritis the clinical features of NoV GII.4 AGE cases were determined in previously presumably naïve young children <2 years of age. NoVs were detected based on polymerase region A-specific PCR and NoV GII.4 genotypes were confirmed using another PCR targeted at capsid region C.

From 2000 to 2002, NoV was associated with 148 cases of AGE in infants. A second NoV infection occurred in 14% of the cases, these were eliminated from the study group. Altogether, 128 primary NoV infections formed the study material. Genogroup GII caused 110 (86%) and genogroup GI 18 (14%) primary infections, with nine different genotypes: GII.4, GII.b, GII.1, GII.2, GII.7, GI.2, GI.3, GI.4, and GI.6. GII.4 was responsible for 31% of NoV AGE cases whereas the second most common genotype was GII.b (26%). The study material was divided into two seasons from July to June from 2000 to 2001 and from 2001 to 2002. There were more NoV AGE cases (75%) in the second year.

According to the Vesikari score, severe NoV AGE was found in 8 cases, moderate AGE in 47 and mild AGE in 70 cases (respectively). The median age of children with NoV AGE was 17 months (interquartile range, 10–19 months). The clinical features of NoV infections were compared between GII.4 and other genotypes. The age distribution between GII.4 (median 18 months) and other genotypes (17 months) was not different. The children infected with GII.4 had a longer duration of diarrhea (median 2 vs. 1, $P = 0.006$), a greater number of diarrheal stools in 24 hours (median 4 vs. 3, $P = 0.003$) and a longer duration of vomiting (median 2 vs. 1, $P = 0.014$). Altogether, severity score points were higher in children infected with GII.4 (median 7, interquartile range 6−9) than in children infected with other genotypes (median 6, interquartile range 4 −7). In conclusion, using the overall severity score, GII.4 was associated with cases of AGE that were more severe than other NoV genotypes ($P = 0.002$).

4.3 Production of the norovirus VLPs for the candidate vaccine (IV)

4.3.1 Expression of capsid protein VLPs

To achieve a high yield of NoV GII.4 VLPs we optimized the conditions for Sf9 cell infections. The best yield was obtained with recombinant BV P2 stock at MOI of 1. The kinetics of GII.4 capsid protein expression in the infected cells was studied to determine the optimal time for harvesting the VLPs. Aliquots of Sf9 cells and the supernatant (100 µl) were collected each day from one to six dpi and examined by SDS-PAGE using PageBlue Protein Staining Solution (Fig. 7). The NoV capsid migrated as a doublet of approximately 58 and 64 kDa under reducing sample buffer conditions and SDS-PAGE. The capsid was detected in the second dpi in the cell lysate; it was released into the supernatant of the infected insect cells starting from the third dpi and it reached the highest level at the sixth dpi, the optimal time for harvesting VLPs. The release of VLPs into the supernatant simplified the purification process.

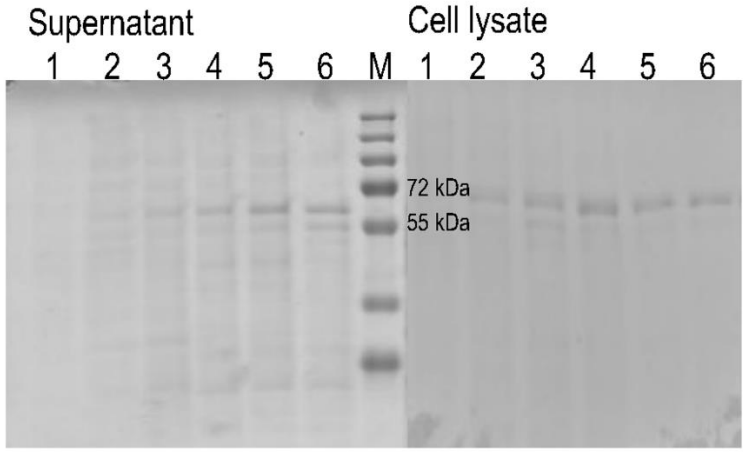

Figure 7. Kinetics of norovirus capsid protein VLP production in Sf9 cells. The supernatant and cell lysate were examined by SDS-PAGE. The day of harvesting (1 to 6 after infection) is indicated above each lane. M = molecular weight marker

4.3.2 Purification and concentration of VLPs

To determine, whether the purification or concentration procedures affect VLPs formation and structure, we used either a sucrose density gradient or CsCl sedimentation. We employed two successive sucrose gradient ultracentrifugation steps, firstly to minimize the presence of soluble capsid protein, and secondly, to obtain purer VLPs. Fractions were collected and analyzed by SDS-PAGE. Examination of the first sucrose fractions showed that apparent peak of the capsid protein migrating to 35% sucrose. These few fractions were pooled and further gradient-purified. Fractions outside the 35% density were not used to ensure that soluble capsid proteins were not collected. In the second sucrose gradient, the capsid protein VLPs migrated to 35% sucrose as well, and eight fractions containing the VLPs were collected. Sucrose was removed by dialysis against PBS, and VLPs were concentrated by dialysis against PEG or ultrafiltration. The purification protocol described above requires several days of laborious work, and, therefore, a less time-consuming procedure was developed. Visible bands of VLPs were collected at the 35% layered sucrose from overnight sucrose gradient ultracentrifugation. VLPs were diluted in PBS and concentrated by ultracentrifugation. In addition, the CsCl sedimentation method was used for the purification of the VLPs and to compare the result to sucrose gradient centrifugation.

The best yield (2–3 mg/200 ml) was obtained after two discontinuous sucrose gradient and ultrafiltration purification procedures. Although the starting quantities of the VLPs were similar in each purification protocol. In comparison, the discontinuous sucrose gradient method where visible bands of VLPs were collected as described above; yielded ten times fewer NoV capsid VLPs.

4.3.3 Characterization of VLPs

The produced capsid VLPs were viewed on 12% SDS-PAGE and stained with PageBlue. Protein bands corresponding to the size of the NoV capsid without degradation products were detected from each purification procedure. However, VLPs purified by the CsCl sedimentation did not display typical NoV doublet bands,

and the VLP preparation concentrated with PEG dialysis were visualized poorly in the SDS-PAGE gel.

NoV capsid identity and the antigenicity of the VLPs were studied. Immunoblotting results using human convalescent serum against NoV GII.4 showed similar antigenicity to NoV capsid proteins after each purification procedure, and no degradation products were detected.

The morphology of the NoV VLPs were detected by EM (Fig. 8). Intact and homogeneous NoV VLPs approximately 38 nm in size were obtained after discontinuous sucrose gradient centrifugation followed by ultrafiltration or ultracentrifugation (Fig. 8B). The visible band of capsid VLPs was collected after sucrose gradient ultracentrifugation (Fig. 8C). These VLPs showed a similar morphology to VLPs purified with two discontinuous sucrose gradient ultracentrifugations. In comparison, CsCl purification (Fig. 8D) and the concentration of VLPs with dialysis against PEG (Fig. 8A) resulted in aggregated, broken VLPs of heterogeneous size.

Therefore, those VLPs purified with discontinuous sucrose gradient centrifugation and concentrated with ultrafiltration resulted in the best quality and yield and they were selected for further use.

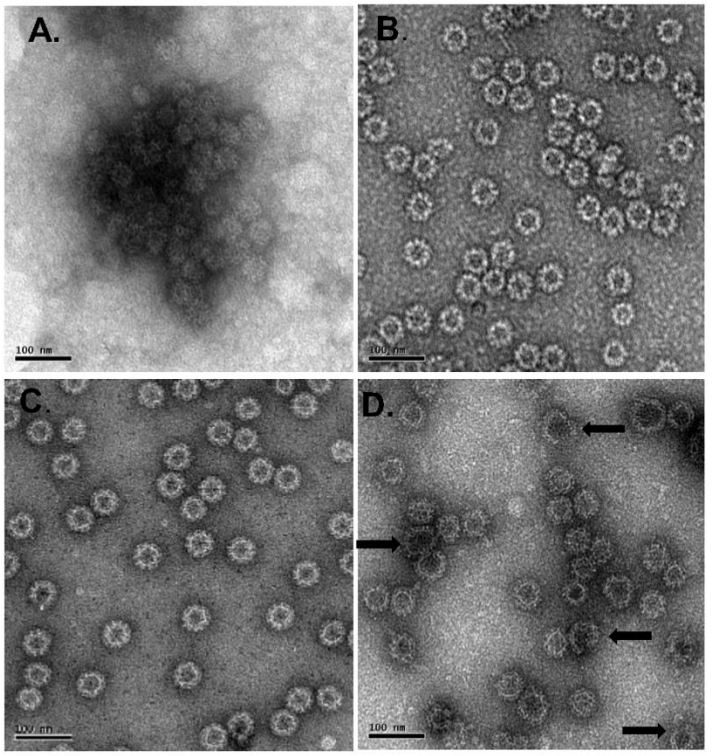

Figure 8. Electron microscopy analyses after several purification procedures. VLPs were purified by two discontinuous sucrose gradient ultracentrifugation and concentration by dialysis against PEG (A) or by ultrafiltration (B). VLPs after purification by discontinuous sucrose gradient ultracentrifugation and concentration by ultracentrifugation (C) or by CsCl cushion and concentration by ultrafiltration (D). The arrows indicates the broken VLPs.

4.3.4 Stability and functionality of sucrose gradient-purified VLPs

NoV VLPs were treated under different conditions and further analyzed by SDS-PAGE, EM and the HBGA binding system. VLPs were stable after 12 months at 4 °C in PBS, pH 7.4 and withstood the sterile filtration conditions using 0.22 μm filters. The stability at RT (23 °C) was confirmed for seven days. VLP morphology was disrupted by heat treatment at 60 °C for 1 h. After the heat treatment, the NoV capsid proteins remained a specific size (58 and 64 kDa), but the VLPs' structures were broken.

The conformational binding sites and VLP functionality were determined by an HBGA binding assay. The results showed that the sucrose gradient-purified VLPs had the best binding capacity, whereas the CsCl sedimentation resulted in a lower binding capacity. However, the binding capacity of the heat-treated VLPs was the lowest. The binding is clearly dependent on the morphology and intact structure of the VLPs where the conformational binding sites remain stable.

4.4 Contaminations in gradient-purified norovirus VLPs (V)

The impurities related to the BES in the NoV VLPs after purification by sucrose gradient centrifugation were determined with various analyses. The SDS-PAGE gel showed a weak band above NoV capsid proteins in the NoV VLP preparation and a similar band in BV mock preparation, corresponding to the size of the BV envelope gp64 protein. Immunoblotting using BV gp64-monoclonal antibody showed BV gp64 protein in the VLP preparation, and EM analysis showed residual BV with VLPs.

4.5 Anion exchange chromatography (V)

To further purify NoV VLP preparations anion exchange chromatography was used. The mock BV and NoV VLP preparations were treated under identical conditions. The fractions from the first anion exchange chromatography yielded NoV VLPs, which eluted three fractions earlier than the BV gp64 protein detected by the SDS-PAGE gel. These fractions were free of BV gp64 protein in the immunoblotting analysis using anti-BV gp64-specific monoclonal Abs. For further purification, the fractions were pooled and applied to a second fresh column. Fractions from the second ion exchange column that were identical to those as in the first column were collected and analyzed by immunoblotting. The immunoblotting result confirmed the purity of the fractions. Therefore, the fractions were pooled, dialyzed against PBS and sterile-filtered. The best purity and yield was achieved with 50 mM Na_2HPO_4-0.5M NaCl buffer, and the optimal amount of protein was 500 μg/column.

4.5.1 Purity and functionality of the VLPs

From the starting material of 2 mg crude purified VLPs we obtained 0.226 mg of highly purified NoV VLPs by anion exchange chromatography. The purity of the VLPs was analyzed using several methods as shown in Table 5. The identity of VLPs was detected by immunoblotting using NoV GII.4-specific monoclonal Abs. The results showed doublets that are typical for the NoV capsid protein, and no degradation products were detected. The immunoblotting analysis with BV gp64-specific monoclonal Abs showed that NoV VLP preparations were devoid of BV. Densitometric analysis confirmed the >90% purity of NoV VLP preparations.

The possible presence of live BV among VLPs was also determined. The mock BV preparation included live BVs (~10^9 pfu/ml). By contrast, the chromatographically purified NoV VLP preparation completely lacked live BV, as determined by a BacPak Rapid Titer Kit. The total DNA amount in the chromatographically purified NoV VLP preparation was quantified. Some DNA was detected, but in low concentration (0.41 ng/μl). Further, the genomic BV DNA was also quantified and a standard curve was created according to the BacPak control BV DNA template (detection range from 1.4 x 10^0 to 1.4 x 10^8 copies/μl). The chromatographically purified VLPs contained few copies of BV DNA. The amount of BV DNA was calculated to be <0.001 pg/μl. The structural characteristics of the purified VLPs were studied with EM (Fig. 9). The structure, morphological integrity and the typical shape of the VLPs were maintained as earlier detected from crude purified VLPs after the chromatography purifications. BV was no longer detected by EM in the VLP samples.

The pure and sterile NoV VLP preparation was shown to be stable after at least 5 months at 4 °C in PBS, pH 7.4. The endotoxin level was measured from VLP preparations to be under 0.1 EU/10 μg of protein; this is far below the international standard of ≤30 EU/20 μg.

The immunogenicity of the purified VLPs was also determined. BALB/c mice were immunized with pure NoV GII.4 VLPs without external adjuvants. Both immunizing doses (1 μg and 10 μg) induced an NoV GII.4-specific serum Ab response. The IgG response was already high after the first immunization but increased after the second immunization at 3 weeks. VLP-specific IgG1 and IgG2a Ab levels were similar in each of the test groups, demonstrating balanced Th1 and Th2a type responses induce by chromatographically purified NoV VLPs.

Table 5. Specification of impurities in the chromatographically purified norovirus GII.4 capsid VLP preparation.

Assay	Specification
Pierce BCA (protein concentration)	0.452 mg/ml
Yield*	0.226 mg
SDS-PAGE and Western blot (identity)	58–64kD protein
Densitometric analysis (purity)	>90%
BacPAK Rapid Titer kit (live BV detection)	0 pfu/ml
Electron microscopy (morphology)	VLPs (~38 nm)
Quant-it dsDNA kit (DNA content)	0.41 ng/μl
BacPAK qPCR kit (BV DNA)	3.31 copies/μl(<0.001 pg/μl)
Limulus amebocyte lysate assay (endotoxin)	<0.1 EU/10 μg of protein
Sterility test (bioburden)	No growth observed

* Total yield after chromatographic purification of 2 mg starting material

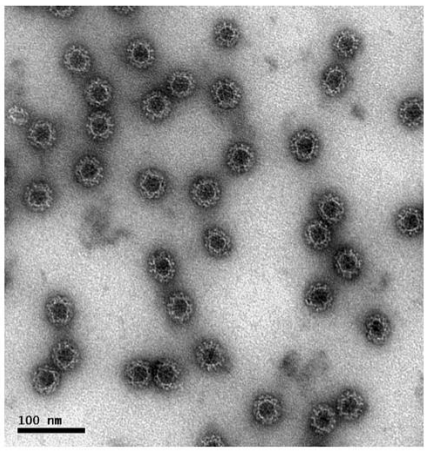

Figure 9. EM of chromatographically purified norovirus VLPs.

4.6 Genetic analyses of norovirus GII.4 variants (III)

4.6.1 Alignment of the P2 domain of norovirus GII.4 variants

Capsid P2 domain sequences of GII.4 variants found in Finland from 1998 to 2013 were aligned. The antigenic similarity and drift of the blockade epitopes (A–E) between various GII.4 strains, especially at the three confirmed epitopes, A, D and E; and the two predicted epitopes, B and C (21,173,189,190); are shown in Figure 10.

In general, the results confirmed that the common changes in the P2 domain also occurred in GII.4 variants found in sporadic cases of pediatric AGE in Finland. As seen in Figure 10, 43 variable sites were detected in the P2 domain. Three interesting amino acid sites (A, D, and E) that have an impact on the HBGA binding and interaction with neutralizing Abs were previously identified: epitope A (aa 294, 296–298, 368, 372), epitope D (aa 393–395) and epitope E (aa 407, 412, 423) (Lindesmith, Donaldson & Baric 2011, Lindesmith et al. 2012, Debbink et al. 2012). The amino acid changes in these sites coincided with the epidemic waves of GII.4 variants in sporadic AGE cases.

The three most recent NoV variants (2008, 2010, 2012) closely matched each other at the site of epitope A. However, epitopes B, C, and D seemed to follow previously circulating variants, but they had amino acid substitutions compared to the precursor strain. Epitope E seems to vary in every epidemic strain. The amino acid sequences between GII.4 VLP (isolated in 1999) and novel GII.4 variants at the variable sites appeared to change without any particular direction, and the previous changes rarely reappeared.

The P2 domain sequence of GII.4 from the VLP candidate vaccine was compared to the GII.4 strains that circulated in Finland between 1998 to 2013 (Fig. 10). Candidate vaccine sequence is from 1999. The 2012 strain has more amino acid changes in the P2 domain than the 2010 strain (31 aa, vs. 26 aa) compared to the GII.4 VLP strain. A comparison of blockade epitopes between the VLP-candidate vaccine strain and the novel 2010 and 2012 strains showed that amino acid 296 in epitope A has remained the same during these years. In addition, the amino acid at position 382, which also belongs to epitope B, is identical in the 2010 and 2012 strains and the precursor VLP strain, but other epitopes have changed over the years.

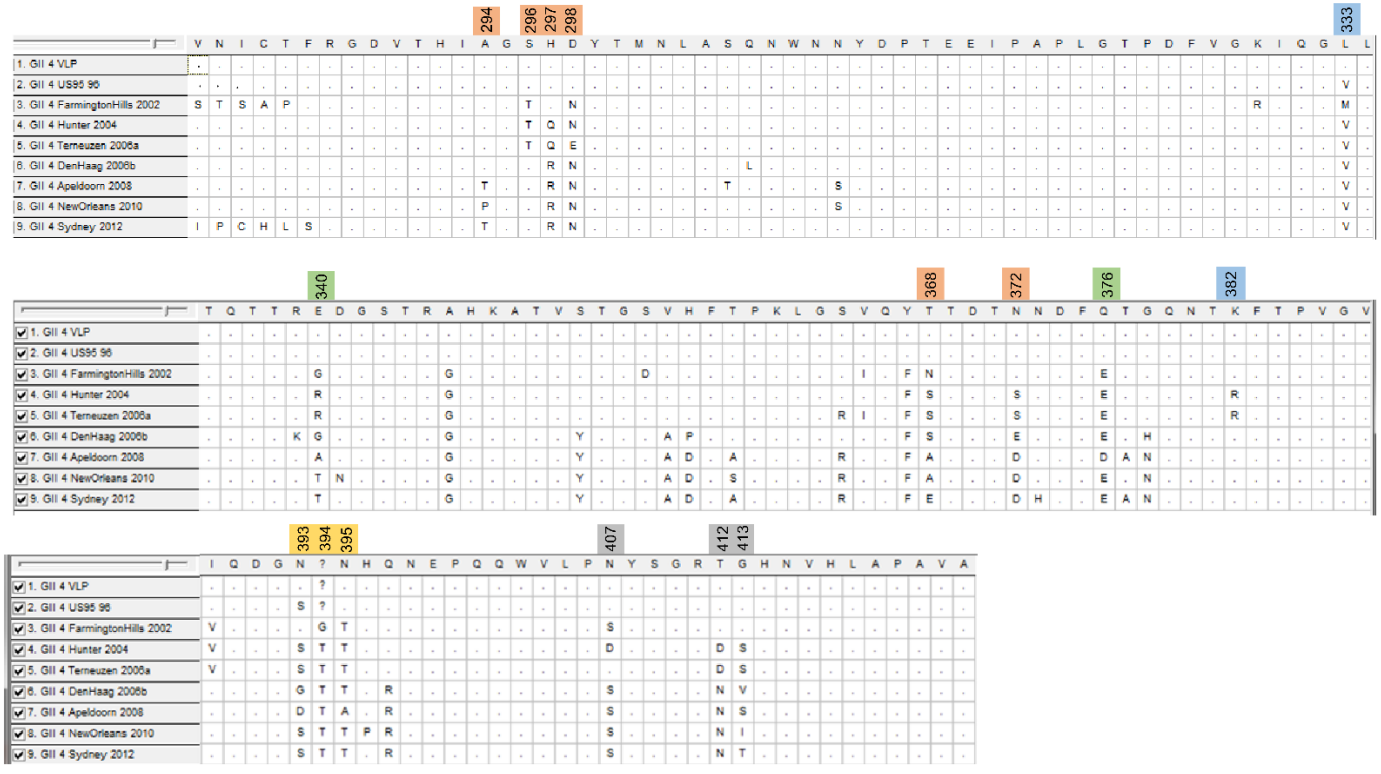

Figure 10. Comparison of the amino acid sequence of the P2 domain (281–423 aa) from norovirus GII.4 strains. The most common strains from GII.4 variants found in non-outbreak norovirus cases were compared with the sequence of the GII.4 VLP that was used as a reference strain. The sequence from GII.4 VLP is at the top of the alignment. Dots indicate sequence identity, and amino acids changes are shown. Question marks indicate missing amino acids. Epitope A: orange; Epitope B: blue; Epitope C: green; Epitope D: yellow; and Epitope E: grey

4.6.2 Phylogenetic analysis of norovirus GII.4 variants

The genetic distances were calculated between NoV GII.4 variants that circulated in Finland from 1998 to 2013. The phylogenetic tree was created from the alignment of 9 amino acid sequences in the GII.4 variants' P domain (aa 142); the tree also includes the GII.4 VLP strain (Fig. 11). However, the genetic distance between 2006a and 2006b was surprisingly long. The 2006a strains sublineaged with the 2002 and 2004 strains whereas the 2006b strains associated closely with the novel strains 2008, 2010, and 2012. The GII.4 VLP was linked to the 1996 strain.

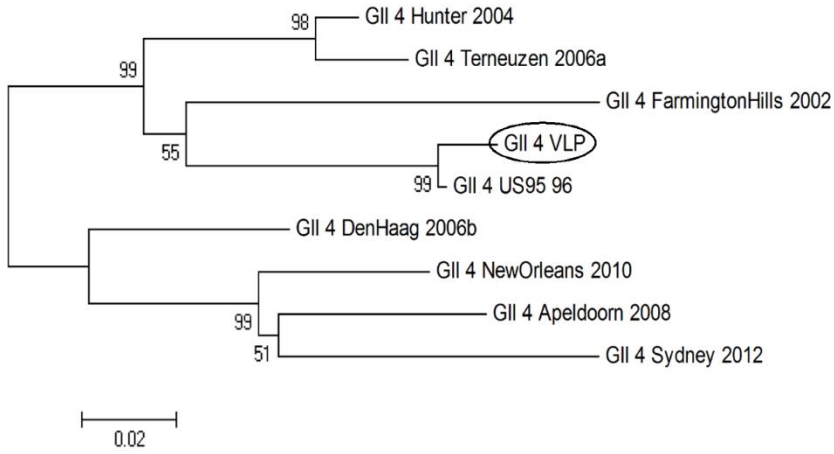

Figure 11. Phylogenetic tree of amino acid sequences in the P2 domain (aa 142). The norovirus GII.4 strains circulated in Finnish children from 1998 to 2013. The nomenclature of GII.4 strains was appointed according to FBVE reference strains. GII.4 VLP sequence is circled. The scale bar represents the number of the amino acid substitutions per site.

5 Discussion

5.1 Sporadic norovirus AGE cases in children (I)

After RV, NoV is the second most common cause of sporadic viral gastroenteritis in children worldwide (1-3). Community-based NoV AGE cases in children were first reported in Tampere using material from the prospective follow-up for AGE of children participating in the RRV-TV vaccine trial in Finland in 1993–95. This study was the first systematic survey in children using RT-PCR as a primary detection method in sporadic childhood AGE. The finding was that 20% of children under two years of age in the community acquired AGE were associated with NoV. (3) The specimens from 1993–1995 were analyzed at the polymerase region with Np110-primers (4). The figure of 20% may be an under-estimation since some cases may not have been detected using these primers, as suggested by Vinje et al. (11).

The RRV-TV vaccine trial and other RV vaccine trials conducted in Finland with fecal specimens collected from all AGE cases provided a good opportunity to investigate the etiological role of NoV in the community-based AGE among children. Primer selection has been challenging. RT-PCR, which detects the polymerase genotypes of NoVs, is able to detect the widest diversity of NoV strains (261). In addition, it is a good diagnostic method for NoVs, allowing genotyping determination from the polymerase region, which is the conserved region in the NoV genome (12). Detection of a large number of NoV-positive specimens, first from the polymerase region, followed by RT-PCR from the capsid region, gave us the unique opportunity to investigate NoV genotypes as determined by both regions. The genotype determined by the polymerase and capsid region varies. Capsid region C was used, since this region is the most conserved on the NoV capsid region for the detection of a large number of samples. However, a recent study has shown that NoV genotype classification should be done using the polymerase and whole capsid VP1 sequences (14).

In this study, NoV was detected by the polymerase region from all specimens collected between 1993–2007. Altogether, 4727 fecal specimens were analyzed and 1172 were NoV-positive. About 25% of children had NoV AGE by the age of three years. In comparison, the incidence of NoV infections was 21% in children in a

prospective surveillance study in three states in the US (237). In Beijing, NoV-positive cases were found in 16% of infants (272), and in Sweden in 15% of AGE cases in children under fifteen years of age (273).

The NoV seasonal peak occurred from November to April. NoV AGE cases in the community appear earlier compared to RV AGE cases (3,274). Why NoVs occur especially in colder months is not well known. The seasonal activity of infectious agents such as RV, influenza, and poliovirus are linked to changes in humidity and temperature, which facilitate viral persistence (275-278). Increased humidity may facilitate NoV transmission from vomit as an aerosol. In winter months, children are in daycare centers and thus in close contact with each other, which facilitates NoV transmission. In winter, diminished UV light in temperate regions reduces vitamin D synthesis, which may reduce immune response (279) and lead to increased susceptibility to infections. Higher exposure to UV light may decrease NoV persistence on surfaces and in the environment in summer-time and it may decrease indirect transmission of NoVs. The temperature is also one factor influencing the infectivity of NoVs. The MuNoV study showed that virus particles are more stable at a lower temperature (+4 ºC) (280). Altogether, the seasonality of NoV is influenced by several factors, such as humidity, UV light, temperature, crowding, and host resistance.

5.1.1 Norovirus polymerase genotypes (I)

NoV AGE cases in children were mainly caused by the genogroup GII (96%). This figure is even higher than in the original study by Pang et al. from Finland in 1993–1995, where GII accounted for 88% of NoV positive cases (3). Worldwide, NoV GII has been the most prevalent genogroup, accounting for 96% of sporadic NoV infections in children from 2004 to 2012. GII.4 accounted for 62% of cases worldwide and; the second most common polymerase genotype was GII.b (14%) (19). However, in our material GII.b was the fifth most common genotype (7%). GII.b caused AGE in children in Sweden from 2000 to 2003, and it was the most prevalent strain in the first study season (2000–2001) (273). GII.b dominated in Finland between 2003 to 2004. Worldwide, the third most common genotype has been GII.12 (19). We did not observe this genotype in Finland between 1993 to 2007. Other circulated genotypes (GII.7, GII.2, GII.3 and GII.1) were observed in chidlren, and these findings are comparable to the results worldwide (19).

GI was detected in 4% of NoV AGE cases and GI.3 was the most common genotype among genogroup GI infections. In a study by Maunula et al., GI.3 accounted for 13% of Finnish outbreaks and GI.3 was the most prevalent genotype of GI from 1998 to 2002 (281). GI rarely causes sporadic AGE in children but it is a common cause of water- and foodborne outbreaks (165,281).

5.1.2 Norovirus GII.4 variants (I,III)

In this study we show that a high GII.4 prevalence in sporadic AGE cases in children and in epidemic years coincides with the emergence of new GII.4 variants. In eleven seasons (1998–2013) six GII.4 variants were associated with epidemic years of NoV in children. These results match earlier findings from NoV outbreaks well. The first GII.4 variant, 1996, was detected in 1995 (175,282). This variant was common in Finnish children from 1998 to 2003 and was associated with the peak season 1998–1999. A new variant, 2002, caused an unusual epidemic peak in the summer and autumn months in Europe (72). In our study, variant 2002 was circulating in Finland from 2000 to 2007, but it was associated with an epidemic peak in 2002–2003. The next variant, 2004, caused epidemics in Europe (70) but it was rarely detected in Finland. Variant 2004 may have circulated in Finland between 2004-2005 and 2005-2006 seasons, but we did not have data from these periods. In early 2006, two variants, namely 2006a and 2006b, emerged (70). Variant 2006b was more common in sporadic NoV AGE cases in hospitalized children. Reports from Canada and Japan also showed a high prevalence of the 2006b variant (283,284). Variant 2008 was detected only in three of our NoV AGE cases. Similar findings were reported from France and Korea where variant 2008 circulated but did not cause an epidemic (285,286). A new variant, 2010, was first reported in autumn 2009 in the USA (178). We detected variant 2010 at a lower rate compared to the earlier variant, 2006b, in hospitalized children. The most recent variant, 2012, circulated in Finnish children in the season 2012–2013. Altogether, all the variants that have caused epidemics and outbreaks worldwide were also detected in this study of sporadic (non-outbreak) cases of NoV AGE in young children. The simultaneous occurrence of the same variants in outbreaks and sporadic cases does not allow us to draw conclusions as to which came first.

5.1.3 Clinical features of norovirus infection (II)

NoV and RV cause severe gastroenteritis symptoms in small children and the clinical picture of NoV and RV is very similar. NoV gastroenteritis has been associated with a higher rate of vomiting but less diarrhea and lower fever compared to RV AGE. In addition, the overall duration of NoV AGE is shorter than RV AGE. The onset of NoV AGE symptoms generally starts with nausea and vomiting, followed by diarrhea and fever. (3) The median Vesikari severity score for RV was 10 points; the score for NoV was 8 points, followed by other less common AGE viruses such as adenovirus (7 points), sapovirus (6 points) and astrovirus (5 points) (132). The incidence of RV AGE has decreased dramatically as a result of universal RV vaccination. NoVs have thus become the most important pathogen of severe AGE in young children. It has been proposed that the severity of NoV AGE is associated with the specific NoV genotype GII.4 (121,287). Previous studies have shown that NoV GII.4 is the most common cause of NoV AGE cases requiring hospital admission in children (1,6,271).

A study by Friesema et al. has shown a difference in clinical features between NoV genotypes in adults (287). Our aim was to study the severity of NoV GII.4 in comparison to other NoV genotypes in those sporadic cases where NoV AGE in children that likely underwent a primary infection. We did not have serum samples to study the presence of pre-existing Abs, but when children were enrolled to RV vaccine trial, they were 36 weeks of age and we could assume that in most cases, the NoV AGE in these children was a primary NoV infection.

We were the first to show that in naïve children, NoV GII.4 caused a longer duration of diarrhea and vomiting and a greater number of stools in 24 hours than other NoV genotypes. The reason for a more severe clinical source is not known, but perhaps GII.4 strain is capable of replicating at a higher rate in host cells because it can bind to wider a range of HBGAs than other genotypes (185,186). The prolonged duration of symptoms of NoV GII.4 in adults was shown to be associated with a higher fecal concentration of NoVs (287-289). Altogether, GII.4 may have greater inherent virulence than other NoV genotypes.

5.2 Norovirus VLP production as a candidate vaccine (VI-V)

BES is highly effective for the production of recombinant capsid proteins and the method has been extensively used for producing VLPs from several viruses

(241,290) including NoV (27,291). NoV VLPs produced by BES have been used in studies of protein interactions (95), virus assembly (58,263), diagnostic serological assays (193), and vaccines (30,292). The target for all these applications is high quality VLPs. We compared different purification and concentration methods for the NoV GII.4 VLP candidate vaccine in terms of purity, integrity, morphology, antigenicity, and functionality.

5.2.1 Upstream process of norovirus VLPs

To obtain a high yield of NoV VLP, the MOI and the time of harvesting the VLPs was optimized. The specific MOI for production depends on the target product and the strategy of production. In our study, we used a MOI of 1 but a wide variety of MOIs have been used by others (63,293,294). The highest yield of VLPs in the supernatant was on day 6 of postinfection. Most of the other investigators have harvested the VLPs earlier (27,63,294)

5.2.2 Downstream process of norovirus VLPs

VLPs purified with different methods showed similar migration and appearance patterns on SDS-PAGE gels. The identity and antigenicity were confirmed with immunoblotting using a human convalescent serum collected from a person with the NoV GII.4 infection. The results also showed that the antigenicity of the VLP capsid proteins was preserved equally well for all purification products.

After two discontinuous sucrose gradient purifications and concentration by ultrafiltration we obtained a remarkably high yield of VLPs, compared to others (63,294). We assume that we did not loose much of VLPs during the purification process.

The morphological integrity and homogeneity of VLPs were analyzed by EM. The VLPs obtained from discontinuous sucrose density gradients concentrated by either ultracentrifugation or ultrafiltration were morphologically homogenous and intact and typical in size (38 nm). CsCl purification resulted in broken VLPs and VLPs of heterogeneous size. As described earlier, CsCl purification has an effect on the morphological appearance of VLPs (215). However, the poorest morphology and aggregation of VLPs were detected after concentration of VLPs by dialysis against PEG. The reason for this may be that the PEG leaked through the dialysis membrane, interfering with the following applications for VLPs (265).

Our results show that SDS-PAGE, immunoblotting or EM results alone or in combination cannot confirm the native quaternary structure integrity of purified NoV VLPs. This issue was addressed by testing the ability of the purified VLPs to bind to a putative NoV GII.4 receptor H-type-3 carbohydrate antigen (193). Intact binding capability is essential when using VLPs to study NoV-specific human or animal serum for the blocking of receptor interactions (95). Our data clearly demonstrated that the purification process affects the receptor-binding functionality of the VLPs. VLPs purified by CsCl appeared highly intact by EM but the HBGA binding, compared with the sucrose density gradient-purified VLPs, showed that the binding intensity of CsCl-purified VLPs was diminished. Others have also shown that CsCl purification affects the functionality of VLPs (215). It may be that CsCl partially denatures VLPs. We also showed that heat-treated VLPs without an intact morphology retain binding capacity but with diminished binding intensity. From these results, we concluded that careful optimization of the purification method for NoV VLPs used in diagnostic serological assays, blocking assays and, particularly, VLP vaccine production, is necessary for high quality VLPs.

As a result of these studies we present a purification system that resulted in high quality NoV VLPs in terms of yield, functionality, stability, and morphology, and it maintained the intact VLPs icosahedral capsid structure (at least up to 12 months at 4 ºC). Even though it has been suggested that VLPs should also contain the VP2 minor capsid protein to stabilize the VLP structure (263), we suggest that VLPs expressed from the VP1 major capsid protein alone are stable after the optimal purification and concentration procedures.

5.2.3 Residual baculovirus in VLP preparation

NoV VLP purification by a conventional method is the most commonly used method in many laboratories (190,217,295). To develop a recombinant vaccine for commercial use, the preparation must be free of any genetic material and impurities related to the expression system. BVs are expressed in high titers in suspension culture (213,223). In most cases, the conventional purification methods do not discriminate VLPs and BVs (213). As an indicator of the presence of BV we detected BV gp64 in the VLP preparations purified by density gradient. The amount of BV gp64 protein in VLP preparation was approximately 10% as judged by densitometric analysis. BV gp64 has an adjuvant effect and it has mannose-binding residues which are expressed on the surface of antigen-presenting cells (296,297). The gp64 protein

has been shown to have an enhancing effect on immune response-recognizing Toll-like receptors and activating inflammatory cytokine (tumor necrosis factor-α, interleukin-6) production (297,298). It has also been shown that BV DNA induces proinflammatory cytokines production as well as the adjuvant properties of BVs (299-301). In addition, live BV activates innate immunity (296,302).

There are a number of available methods to inactivate live BV, such as pasteurization, treatment with non-ionic detergents, and DNA alkylation (226). However these inactivation methods may negatively influence the VLP structures, whereas chromatographic purification allows high resolution separation without such effects (300,303). Several steps are required to discriminate VLPs from BV by chromatography, and the disadvantage is that the repeated purification procedures can affect the conformation of VLPs (225,304-306). Therefore, we purified VLPs further using anion exchange chromatography. Anion exchange chromatography is based on the binding of charged proteins and should remove the impurities such as BV proteins and DNAs (225,262,303,307). SDS-PAGE and immunoblotting resulted followed by densitometric analyses indicated highly purified NoV VLPs (>90% purity) without contaminating BV gp64 protein. Chromatographically purified VLPs were comparable to conventional purified VLPs in terms of icosahedral capsid structure and stability. We quantitated the number of copies of the BV DNA by Q-PCR and observed that the dsDNA content in pure NoV VLP preparations was low (0.9 ng/1 μg dose). The acceptable threshold value of DNA content for a VLP vaccine is 10 ng/dose (308). Thus, we demonstrated that neither live BV nor a significant quantity of BV DNA was found in the purified NoV VLP preparation.

Purified NoV VLP preparations were highly immunogenic. Our immunization data clearly demonstrates that pure NoV VLPs without BV induce a strong IgG immune response and balanced IgG-subtype responses (reflect to Th1- and Th2a-type immune responses), and the immune responses were comparable to those seen by conventionally purified VLPs (231,232). Others have also shown that VLP without an adjuvant induces strong B- and T-cell responses (209,309).

5.3 Genetic analyses of norovirus GII.4 variants (III)

A number of studies have identified changes in the blockade epitopes of GII.4 variants either from NoV outbreaks or from reference strains from Genbank (23,181,190). We were interested in the phylogenetic analyses of GII.4 variants and

identification of epitope changes in GII.4 strains found in sporadic (non-outbreak) NoV GII.4 AGE cases in Finnish children.

Two variants of GII.4 (2006a and 2006b) emerged simultaneously worldwide (177,310). However, they variants are highly different. Variant 2006a was associated with the ancestor variant 2004 whereas variant 2006b was later linked to recent variants 2010 and 2012 in pediatric AGE cases in Finland, and also in Canada and the rest of Europe (179,183,283). We assume that a higher number of amino acid changes in the NoV P2 domain allowed the virus to avoid pre-existing immunity. Therefore, the 2006b variant occurred more often and caused more pediatric NoV AGE cases in Finland.

Previous studies from outbreaks have identified amino acid changes in the blockade epitopes, and we detected similar changes in nine GII.4 strains from sporadic NoV AGE cases. The blockade epitopes A, D, and E vary between epidemic strains, whereas epitopes B and C do not (173,181,182,236). Changes in these epitopes result in new variants that could escape from herd immunity (20,23,182). However, these changes in the epitopes do not reappear. Therefore, it is impossible to predict amino acid changes prospectively, as is the case for influenza viruses. The amino acid changes between the epitopes do not completely explain why some variants are epidemiologically more significant than others. Siebenga et al. speculated that it could be the result of herd immunity or different binding patterns of GII.4 variants (70,181,311). Another possibility is the occurrence of seeding events through water- and food-related outbreaks that are difficult to detect but are highly common (70,312).

We also compared the amino acid sequence of the blockade epitopes of the GII.4 VLP (our candidate NoV vaccine) to circulating strains. All variants have different blockade epitopes compared to the VLP 1999 strain. Nevertheless, mice immunized with 1999-VLP induced cross-blocking Abs to the more recent strains, GII.4 VLP 2010 and 2012 (187,231,232). GII.4 2010 mouse sera induced a moderate Ab blocking response against the 2012 variant but did not block GII.4 1999 VLP-HBGA binding (187) (Malm, unpublished). For comparison, GII.4 1987 monoclonal Abs also blocked the VLP-HBGA interaction of the later circulating strains (1997 and 2002) (189). GII.4 VLP-1987 immunized polyclonal mouse sera did not block the interaction between H-type-3 and GII.4-2006-VLP (190). As others have already suggested (173), we also assume that there are still existing neutralizing epitopes that have remained uncharacterized. However, at present it seems that high levels of cross-blocking Abs induced by our candidate vaccine GII.4 VLP strain should

produce sufficient cross-protective immunity against new variants that have appeared since 1999 to the present.

6 Summary and Conclusions

In this thesis, the overall aim was to evaluate NoV genotype distribution and particularly the role of NoV GII.4 in sporadic NoV AGE cases in Finnish children for the purpose of NoV vaccine development. In addition, the methods for NoV VLP vaccine production were developed. Antigenic changes in circulating NoV GII.4 variants were characterized and compared with the corresponding VLP vaccine candidate derived from 1999.

Study materials of sporadic AGE cases collected from Finnish children between the years 1993 and 2013 were analyzed. NoV GII.4 was the most common genotype already from the beginning of our study period in the community-based materials in children under three years of age. In addition, GII.4 dominated in NoV AGE in hospitalized children in 2006–2013. The NoV epidemic years as seen in Finnish children were associated with the emergence of new variants of GII.4. Six GII.4 variants (1996, 2002, 2006a, 2006b, 2010 and 2012) were responsible for different epidemic peak years in Finland.

The dominance of NoV GII.4 in the study material and earlier reports from Tampere University Hospital lead to the assumption that GII.4 may cause a more severe form of the disease. The finding in this study of greater clinical severity of GII.4 in children with a primary NoV infection resulted in the hypothesis that GII.4 may be inherently more virulent than other genotypes. A possible explanation might be the greater variability of HBGA receptors for GII.4 than other genotypes.

Accordingly, a NoV vaccine for children should contain the GII.4 genotype. To produce high quality GII.4 VLPs we compared conventional purification and concentration procedures for yield, morphology, immunogenicity, antigenicity and functionality. Two discontinuous sucrose gradient ultracentrifugations and concentration with ultrafiltration resulted in VLPs with an icosahedral capsid structure and good receptor binding capacity. These VLPs were highly stable (12 months at 4 °C and up to 7 days at RT). After conventional purification methods, VLP preparations still contained impurities (live BV, BV DNA, BV protein) derived from BES. VLPs were purified further with anion exchange chromatography. These VLPs preserved their intact morphology, antigenicity and immunogenicity. We suggest that this small-scale laboratory purification procedure allows a good layout

for large scale production for NoV VLPs for use as a candidate vaccine in clinical trials.

Antigenic variation of the GII.4 P2 domain between epidemic GII.4 variants is a response of the virus to host immunity. Natural NoV infection induces type-specific and rather short immunity. In contrast to immunity following natural infection, a NoV vaccine should be able to induce high levels of cross-protective Abs sufficient for durable protection across GII.4 variants. A NoV vaccine should contain viruses from both genogroups GI and GII. As described earlier, in here the GI.3 was the most prevalent genotype of genogroup GI, and was thus selected to be a part of our candidate NoV vaccine. Knowledge of the production and purification of GII.4 VLP has been used to develop VLPs of other NoV genotypes. The GII.4 and GI.3 NoV VLPs were combined with RV VP6 to make a NoV-RV trivalent vaccine. This vaccine is presently in preclinical studies.

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Norovirus genotypes in endemic acute gastroenteritis of infants and children in Finland between 1994 and 2007

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SUMMARY

Noroviruses are, after rotaviruses, the second most common causative agents of acute gastroenteritis in young children. We studied norovirus genotypes in faecal specimens collected from Finnish children followed-up prospectively in rotavirus vaccine trials. Almost 5000 faecal specimens collected from cases of acute gastroenteritis were examined using reverse transcriptase–PCR. A total of 1172 cases (25% of all acute gastroenteritis) were associated with noroviruses. Of these, 96% were genogroup GII. GII.4 was the most common genotype (46%) throughout the study period but the proportion of this genotype varied in different norovirus epidemic seasons. Additional norovirus genotypes detected were: GII.7 (15%), GII.3 (14%), GII.1 (9%), GII.b (7%), GII.2 (3%), and GI.3 (2%). GII.4 dominated during the following years: 1998–1999 (75%), 2002–2003 (88%) and 2006–2007 (98%) while recombinant genotype GII.b was dominant between 2003 and 2004 (83%). In conclusion, genotypes GII.4 and GIIb have emerged as predominant norovirus genotypes in endemic gastroenteritis affecting young infants and children in Finland.

Key words: Gastroenteritis, Norwalk agent and related viruses, virology.

INTRODUCTION

Noroviruses (NoVs) are not only the leading causative agents of outbreaks of acute viral gastroenteritis worldwide in people of all ages, but also the second most common viral aetiological agents of severe childhood gastroenteritis after rotavirus [1, 2]. Patel and co-workers [1] estimated that in children aged <5 years residing in resource-rich countries, NoVs cause ~900 000 episodes of gastroenteritis necessitating a clinic visit, compared with resource-poor countries in which NoVs may cause more than one million hospitalizations and up to 200 000 deaths each year.

A high incidence of NoVs (20%) in acute gastroenteritis (AGE) affecting young children was originally reported from Finland when stool specimens collected for rotavirus studies in connection with a rotavirus vaccine efficacy trial in 1993–1995 were also examined for human caliciviruses [3]. While many of the community-acquired NoV cases were mild, some were severe, and NoVs also accounted for about 10% of AGE cases seen in hospital [4]. In the present study, we examined the incidence and genotypes of NoVs in

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Table 1. *Study specimens analysed and the methods used for RNA extraction and NoV genotyping 1993–2007*

Study years	No. of samples	NoV-positive specimen, *N* (%)	Boom's extraction	QIAamp Viral RNA extraction	Region A* genotyping	Region C* genotyping
2007	106	57 (54)	−	+	+	+ (53)†
2002–2004	254	27 (11)	−	+	+	−
2001–2003	569	90 (16)	−	+	+	−
2000–2002	485	146 (31)	+	−	+	+ (140)
1998–2001	1510	438 (29)	−	+	+	−
1997–1999	326	101 (31)	−	+	+	−
1993–1995‡	1477	313 (21)	+	+ (279)	+ (294)	−
Total	4727	1172 (25)	1962	3044	1153	193

−, Not tested; +, tested.

* See Methods section and Figure 1.

† If not all specimens were tested the number in parentheses indicates the number of specimens tested.

‡ Results published in [4].

community-acquired AGE affecting young children during 1993–2007, a period when large numbers of infants and young children participated in several rotavirus vaccine trials in Finland.

Most NoVs infecting humans belong to genogroups GI and GII. GI genogroup is further subdivided into at least eight genotypes and GII genogroup into 17 genotypes [5]. Of the two main genogroups, GII is much more common worldwide [6, 7]. Genotype GII.4 NoVs have predominated since the mid-1990s in the USA, Europe and Oceania, causing 70–80% of all NoV outbreaks in communities, nursing homes, schools, hospitals and cruise ships associated with contaminated food or water [8, 9]. GII.4 has also commonly been detected in endemic infections of children worldwide [10–14]. Of the other genotypes, recombinant strain GIIb especially has also been frequently reported in paediatric NoV infections [11–13, 15].

In the past, NoV genotyping was performed solely on the RNA-dependent RNA polymerase (RdRp) region of open reading frame (ORF) 1 of the single-stranded, positive-sense NoV RNA genome [16]. Later studies showed better segregation of the different strains into their respective genotypes by phylogenetic analysis of nucleotide sequences within the capsid region of ORF2 [17]. However, genotyping based solely on the capsid sequence would miss the naturally occurring recombinant NoVs which cluster into two distinct groups of NoV strains when regions RdRp and capsid are subjected to phylogenetic analysis [18, 19]. In this study, we genotyped NoVs according to polymerase region as well as to the capsid region.

METHODS

Clinical specimens

In total, 4727 faecal specimens were collected from AGE cases in children up to age 3 years participating in seven rotavirus vaccine efficacy trials in several different regions in Finland from 1993 to 2007 [3, 20–23]. The vaccine study protocols and consent forms had been approved by the appropriate ethics committees and patients or legal guardians volunteered for the study after having given informed consent. Stool specimens were collected from children receiving either placebo or rotavirus vaccine whenever an episode of AGE occurred. AGE was typically defined as $\geqslant 3$ looser than normal stools and/or vomiting within any day. Specimens collected within 14 days of the first sample from the same patient were considered to be duplicate samples and excluded from the analysis. The numbers of stool specimens from each year and the genotyping methods applied are described in detail in Table 1. Stool specimens were stored at −20 °C until tested.

RNA extraction

For viral RNA extraction 10% (w/v) stool suspensions were made in phosphate-buffered saline (PBS). Extractions were performed using the QIAamp® Viral RNA Mini kit (Qiagen, Germany) according to the manufacturer's instructions. In some cases (Table 1), RNA was extracted by binding to silica particles in the presence of guanidine thiocyanate as described earlier by Boom *et al*. [24]. Aliquots of

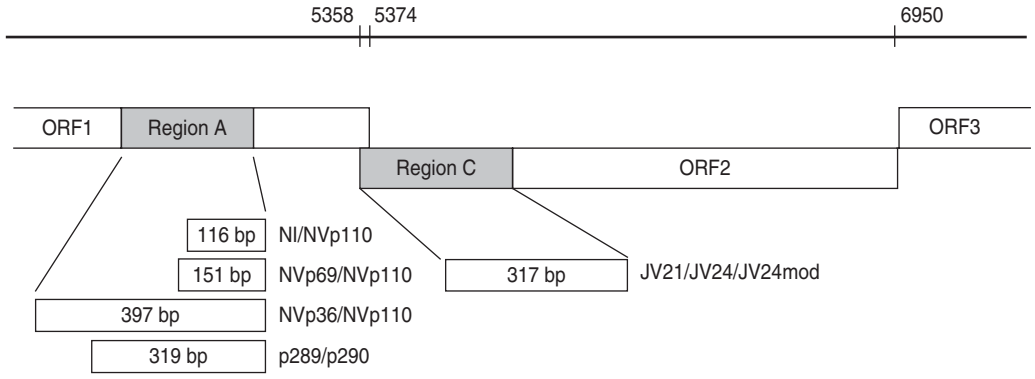

Fig. 1. Schematic presentation of norovirus genomic regions A and C (grey) [17], and RT–PCR primers (NI/NVp69/NVp36/NVp110, p289H,I,IUB/p290H,I,J,K,IUB and JV21/JV24/JV24mod, described in the Methods section) used for reverse transcriptase (RT)–PCR and genotyping. Numbers on the bar refer to the nucleotide positions in Norwalk GI.1 virus genome (GenBank accession no. M87661). RT–PCR amplicon sizes in base pairs are noted in the boxes.

RNA extracts were stored at −70 °C until reverse transcriptase (RT)–PCR testing.

RNA polymerase (region A) RT–PCR

For the specimens collected between 1994 and 1995 the detection of NoVs was performed using primer mixture sets including reverse primer NVp110 and forward primers NI, NVp69 and Np36 (Fig. 1) [25]. These primers detect both genogroup I and II NoVs as well as sapovirus, and amplify 397 bp, 151 bp and 116 bp long PCR products from the RNA-dependent RNA polymerase (RdRp) region. RT–PCR reactions were performed as described previously [3].

For the specimens collected between 1997 and 2007, as well as for 279 samples from 1994 to 1995 (to confirm genotype findings done by the RT–PCR method described above), RNA polymerase region A detection was performed by the RT–PCR method reported by Jiang and co-workers [26], modified by Farkas and colleagues [27]. The primer mixture p289H, I/p290H,I,J,K was used with additional primers p289IUB (reverse: 5′-GATTACTCCARGTGGGAYTCMAC-3′) and p290IUB (forward: 5′-TGACRATKTMATCATCMCCRTA-3′) to improve detection of all genotypes. The RT reaction was performed at 42 °C for 60 min with 2·5 μl RNA added to the RT mixture (total volume 50 μl) containing 22·9 μl sterile water, 1 × GeneAmp PCR buffer (Applied Biosystems, USA), 1·5 mM GeneAmp $MgCl_2$ (Applied Biosystems), 400 μM dNTPs each, 16 ng/μl p289H,I,IUB reverse primer mixture, 10 U RNasin® (Promega, USA) and 70 U M-MLV Reverse Transcriptase RNase H^- enzyme (Promega). Fifty microlitres of PCR reaction mixture consisting of 26·6 μl sterile water, 2 U GoTaq DNA polymerase (Promega), 1 × GoTaq Green buffer (Promega), 0·5 mM $MgCl_2$ (Promega), and a mixture of p290H,I,J,K,IUB forward primers (24 ng/μl) was added to the RT reaction. The 40-cycle PCR was run in a GeneAmp PCR system 9700 or Thermal Cycler 2720 (Applied Biosystems) with the following conditions: primary denaturation at 94 °C for 3 min, denaturation at 94 °C for 30 s, annealing at 42 °C for 1 min 30 s, extension at 72 °C for 1 min and final extension at 72 °C for 10 min. The PCR amplicons were analysed by agarose gel electrophoresis to confirm the correct size of the product. This RT–PCR method for the detection of RdRp of caliciviruses simultaneously detected both NoVs and sapoviruses and amplified a 319-bp amplicon for NoVs and a 331-bp amplicon for sapoviruses. Positive PCR products were stored at −20 °C for sequencing.

Capsid (region C) RT–PCR

To confirm the genotyping by region A, RT–PCR typing targeted at region C (Fig. 1) from the beginning of the capsid region in ORF2 of NoVs was studied for 193 specimens collected between 2000–2002 and 2007 (Table 1). Five microlitres of RNA was first reverse-transcribed as described by Pang *et al.* [28], except that the reaction contained 1 × first-strand buffer (Invitrogen, USA) and the final concentration of dNTPs was 375 μM each. Synthesized cDNA was stored at −20 °C unless used immediately for PCR reaction. A 317-bp fragment was amplified with primers JV21 (reverse), JV24 (forward) [29] and an additional forward primer JV24mod (5′-GTGAATGAAGATGGCGTCGA-3′)

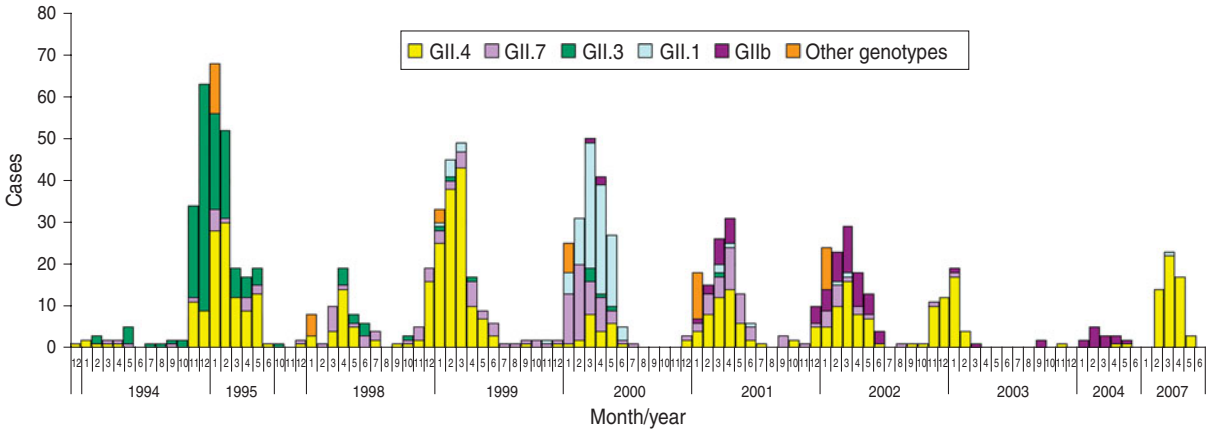

Fig. 2. Norovirus (NoV) seasonality and genotype distribution in Finnish children aged <3 years throughout the study period 1993–2007. From July 1995 to September 1997 and from June 2004 to January 2007 no specimens were collected.

for GII genotypes (Fig. 1). The PCR reaction was performed with 5 μl cDNA added to PCR mixture (total volume 50 μl) consisting of 22·5 μl sterile water, 2·5 U GoTaq DNA polymerase (Promega), 1 × GoTaq Green buffer (Promega), 1 mM $MgCl_2$ (Promega), 200 μM dNTP (Promega) and 4 ng/μl mixture of each of the above primers. The PCR run conditions were identical to those described above for RNA polymerase RT–PCR except that annealing temperature was 49 °C.

Sequencing

All the positive PCR products were excised from the electrophoresis gel, purified with QIAquick® Gel Extraction kit (Qiagen) according to the manufacturer's instructions and sequenced using the Big Dye® Terminator v. 1.1 Cycle Sequencing kit (Applied Biosystems). The primers used for sequencing were identical to those used in the RT–PCRs. Ethanol precipitation purified sequencing PCR products were analysed using an automated sequencer ABI PRISM™ 310 Genetic Analyser (Applied Biosystems).

Sequence analysis and genotyping

Sequences obtained by the ABI PRISM 310 Genetic Analyser were aligned, verified and edited with the program Sequencher™ 4.8 software (Gene Codes Corp., USA). Virus confirmation and genotyping was done using the Food-borne Viruses in Europe network (FBVE) NoV genotyping tool (http://www.rivm.nl/bnwww) and NCBI Blast® programs (http://www.ncbi.nlm.nih.gov/BLAST/, nucleotide blast). In addition, RdRp region A and capsid region C (Fig. 1) sequences of the recombinant strains were re-analysed with the new NoV genotyping tool of FBVE (http://www.rivm.nl; National Institute of Public Health and the Environment, The Netherlands) to confirm the genotyping.

RESULTS

A total of 4727 faecal specimens from AGE cases were studied and 1172 (25%) cases were found to be associated with NoVs, as determined by genotyping according to region A sequences (Table 1). NoV genotypes during the period 1994–2007 by month are shown in Figure 2. The study period covered ten seasons with each season starting in July and ending in June. Of the 1149 genotyped NoV strains, 96% were genogroup GII. GII.4 was the most common genotype throughout the study period (46%), but it appeared in varying proportions in the NoV epidemic seasons (Fig. 2, Table 2). GII.4 was already present in community-acquired cases in 1993–1994 with a 40% share, but dominated in the seasons 1998–1999, 2002–2003 and 2006–2007 (Table 2, values in bold face).

Additional NoV genotypes detected are listed in Table 2. Other genotypes included: GI.6, GI.4, GII.9, GI.2, GIIU, GIId which accounted for <1% each (Fig. 2, Table 2). During the seasons 1999–2000 and 2003–2004, which followed the GII.4 peak seasons, the shares of GII.4 were low (13% and 17%, respectively), and genotypes GII.1, GII.7 and GII.b dominated. In the early study years GII.4 was circulating with GII.3 (1994–1998) and with GII.7 (1994–2002), followed by GII.1 (1999–2000) and

Table 2. *Most commonly found NoV genotypes in sporadic cases of acute gastroenteritis in Finnish children 1993–2007. From July 1995 to September 1997 and from June 2004 to January 2007 there were no specimens available*

Season* …	93–94	94–95	97–98	98–99	99–00	00–01	01–02	02–03	03–04	06–07	
Genotype	Percent of cases per season										Total (%)
GII.4	40	40	47	**76**	13	38	37	**88**	17	**98**	46
GII.7	20	5	22	15	29	27	12	6			15
GII.3	40	51	17	2	3	1					14
GII.1				4	48	3	1			2	9
GII.b					2	12	28	4	83		7
GII.2			5	1		8	12				3
GI.3				2	4	2	3	2			2
Other†		5	9	2	3	9	7				4
N‡	*15*	*279*	*58*	*196*	*197*	*125*	*153*	*51*	*18*	*57*	*1149*

Bold values indicate peak years of GII.4 incidence.
* Season is a time period from July to June.
† Other genotypes detected (GI.6, GI.4, GII.9, GI.2, GIIU, GIId and undetermined).
‡ Total number of NoV-positive specimens (in italics).

GII.b in the later seasons (2000–2007). Overall, of the total ten study seasons GII.4 predominated in seven seasons, while other genotypes predominated only in one season each (Fig. 2, Table 2).

The second most common genotype throughout the 14 study years was GII.7, which appeared in multiple seasons but never dominated (Fig. 2, Table 2). Genotype GII.3 circulated largely in 1994–1995 (40–50%) and 1997–1998 (17%) but was not detected after 2001. Genotype GII.1 was detected only rarely in other seasons, but predominated in 2000. The first recombinant GII.b strains (GII.b–GII.2) appeared in March 2000. GII.b reached a high prevalence in 2001–2002 and was the dominating genotype in 2004. The most common GI strain GI.3 occurred during 1998–2003 with very low prevalence of 1–4%. Only a few AGE cases with other GI strains (GI.6, GI.4, GI.2) were occasionally detected (data not shown). In 1994–2002 there were more than eight different genotypes circulating among the study children, but later, in 2002–2007, the number of different genotypes was at maximum three per season (Table 2).

To confirm whether genotyping by capsid region C would identify identical genotypes as determined by polymerase region A, some of the specimens (years 2000–2002 and 2007, $n=193$) were genotyped for both of these regions (Fig. 1, Table 1). Identical genotyping results were obtained for both regions in all cases, except for the recombinant strains. Altogether 53 recombinant strains were found in 193 double genotyped NoVs in 2001–2002. No recombinant strains were found in 2007, when GII.4 was detected almost exclusively (Tables 1 and 3). RNA polymerase genotype GII.b occurred most commonly with capsid genotype GII.3 (41 cases, 77%) and rarely with GII.1 (three cases, 6%). Polymerase type GII.7 occurred with capsid genotype GII.6 (six cases, 11%) and GII.14 (one case, 2%) (Table 3).

Table 3. *Polymerase (region A) and capsid (region C) genotype combinations of recombinant strains detected in Finnish children in 2001–2002*

Region A	Region C	No. of cases (%)
GII.b	GII.3	41 (77)
GII.b	GII.1	3 (6)
GII.b	—	2 (4)
GII.7	GII.6	6 (11)
GII.7	GII.14	1 (2)

—, Undetermined.

DISCUSSION

Rotavirus vaccination studies conducted in Finland with follow-up AGE specimens offered a unique opportunity to study extensively the prevalence of NoVs in community-acquired AGE in infants and young children. Due to genetic variation in NoVs, primer selection for RT–PCR assays has been challenging.

It is possible that in early study seasons from 1993 to 1995 NoV-positive specimens were missed when we used polymerase region primers including NVp110 [25] which were shown to fail to detect some NoV strains [30]. However, the large number of samples allowed us to draw certain conclusions. The present study showed that in children aged <3 years, NoVs were responsible for about one quarter of AGE cases in the community.

Overall, GII.4 was the most prevalent genotype in young children during the study period 1994–2007, followed by GII.7, GII.3, GII.1 and GII.b. In European outbreaks of AGE in all ages 2001–2006 the most common genotypes were GII.4, GII.7, GII.2 and GII.b [8]. In European outbreaks GII.7 peaked in 2005–2006 [8] and in Finnish outbreaks in 2001 and 2002 [31]. However, in children we had already found GII.7 in earlier seasons (1997–1998 and 1999–2001). Consistent with the observations from outbreaks in Finland and other European countries [8, 11, 31] in winter 2001, GII.b was the third most common genotype, and its relative role increased until winter 2004, when it was the predominant type (83%). In contrast to our observation, in a Canadian study [32] no correlation was found between the NoV strains in the outbreaks and in sporadic childhood AGE, but the follow-up period was only a little over 1 year.

In our study, genogroup GI strains were uncommon and, therefore, appeared to be less common in sporadic cases in children than in outbreaks. In Finnish outbreaks in 1998–2002 GI strains were detected in 13%, but we detected GI in <6% [31]. On the other hand, both in the outbreaks and in our study, GI.3 was the most common GI genotype [31]. In general, GI strains occur throughout the year rather than seasonally, more often in water-mediated rather than person-to-person-mediated outbreaks, and only rarely in sporadic cases of NoV infections [9, 33–35].

The total proportion of GII.4 NoV in young children was 46% for the years 1994–2007 and 52% for 2001–2007. Genotype GII.4 has also been detected as the predominant NoV genotype in paediatric sporadic AGE cases reported elsewhere [11, 13, 14, 36]. The peaks of genotype GII.4 in winters 2002–2003 and 2006–2007 detected in Finland, occurred at the same time as peaks of GII.4 outbreaks elsewhere in Europe and the USA [8, 9]. In 1998–1999 GII.4 was also seen in Finnish outbreaks and this winter was also reported as a GII.4 outbreak peak year in Germany [31, 37]. Furthermore, from 2006 to 2008 GII.4 was found in almost 90% of children hospitalized for NoV AGE (Räsänen *et al.*, unpublished observations). An increase in NoV activity in outbreaks was seen in Finland and many other European countries in 2002 due to the emergence of a new GII.4 variant 2002 [8, 31, 37]. GII.4 variants 2006a and 2006b were responsible for large NoV outbreaks in Finland in winter 2007 [38]. Analyses of genetic variants of GII.4 in Finnish children for the peak years are ongoing and will determine which subtypes were responsible for these peaks.

Naturally occurring recombination events are common in NoVs and the most common recombination site is the ORF1–ORF2 junction localized upstream of the capsid gene [18, 19]. We found 77% of the recombinants in our study consisted of a combination of GII.b–GII.3, which was also the most common recombinant strain elsewhere and emerged recently as a causative agent for many outbreaks in Europe, Australia, and Asia [15, 19, 33]. Genotype GII.b was the most common genotype in Finnish outbreaks in 2001 but was not detected in Finland before January 2001 [34] nor elsewhere in Europe before August 2000 [35, 39]. In the present study six GII.b cases had already been detected in March–April 2000. This raised a new possibility regarding the origin and transmission of the recombinant GII.b strains, which were previously suspected to be in contaminated shellfish in August 2000 [39]. Children with sporadic gastroenteritis could serve as a reservoir for emerging epidemic NoV strains as some NoV strains appeared in children prior to emerging as epidemic outbreak strains [13, 36]. To the best of our knowledge, we report for the first time the recombination of GII-7 polymerase with GII.14 capsid. However, the recombinant cases we described should be verified by sequencing over the ORF1–ORF2 junction region to exclude the possibility of mixed infections of different genotypes.

In conclusion, similar to findings worldwide, GII.4 was found to be the predominant NoV genotype in endemic gastroenteritis in infants and young children in Finland, at least since 1994. Recombinant genotype GII.b was detected with increasing incidence from March 2000 onwards. The shifts in NoV genotypes may be associated with an increase in the clinical significance of NoV AGE in young children overall, and warrant more in-depth studies on GII.4 variants.

ACKNOWLEDGEMENTS

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DECLARATION OF INTEREST

None.

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BRIEF REPORT

Norovirus GII-4 Causes a More Severe Gastroenteritis Than Other Noroviruses in Young Children

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Norovirus (NoV) GII-4 has emerged as the predominant NoV genotype in outbreaks of gastroenteritis worldwide. We determined clinical features of NoV GII-4 associated acute gastroenteritis (AGE) in comparison with AGE associated with other NoV types in infants during seasons 2001 and 2002. During the prospective follow-up period, 128 primary infections of AGE due to NoV were identified in 405 infants; of these, GII-4 was found in 40 cases (31%). NoV GII-4 was associated with longer duration of diarrhea and vomiting than other NoV genotypes, suggesting greater virulence of NoV GII-4.

Norovirus (NoV) is the second most frequen causative agent of acute gastroenteritis (AGE) in young children after rotavirus [1]. NoV AGE in young children is likely to result from primary NoV infection. An early seroprevalence study in Finland found that 49% of infants were infected with NoV by the age of 2 years [2]; some of those NoV infections were clinically manifest, and others were subclinical. In a prospective study of rotavirus vaccine recipients in Finland during 1993–1995, NoV AGE was observed in 20% of the children in a 1-year follow-up study [1]. In this study, genogroup GII accounted for 90% of the cases of NoV AGE [1].

Since 1995, NoV GII-4 genotype has emerged worldwide, displaced other NoV GII genotypes, and caused an increase in overall incidence of NoV infection [3, 4]. The reason for the surge of GII-4 activity is unknown, and 2 questions arise: is GII-4 inherently more virulent than other NoVs, and if so, what is the underlying mechanism? The polymerase region has been identified to be a marker of major NoV-associated gastroenteritis outbreaks (i.e. virulence) [3]; however, changes in the capsid region also affect the antigenicity, host specificity, host cell binding, and virus entry properties and, thereofore, may affect virulence [4].

A confounding factor in studies of virulence of NoV genotypes is pre-existing immunity, which might ameliorate the clinical course of gastroenteritis caused by old NoV genotypes but not by a newly emerged type, such as GII-4. We therefore conducted a comparative study on the clinical severity of AGE caused by NoV GII-4, compared with other genotypes, in young children. We propose that our study cases of NoV AGE in infants likely represent primary NoV infection in previously naive children. Our study material therefore provides a unique opportunity to examine the relationship between the clinical features of our study cases and the genotypes of causative NoVs and, in the case of GII-4, variants of GII-4 without the influence of pre-existing immunity on the clinical severity of episodes.

METHODS

The clinical material was originally collected for an efficacy trial of rotavirus vaccine RIX4414 during 2000–2002 in Finland, as described elsewhere [5]. The vaccine study protocol and consent forms were approved by the appropriate ethics committees and patients or legal guardians volunteering for the study after informed consent was provided. Children were vaccinated at 2 and 4 months of age and followed up for 2 rotavirus winter-epidemic seasons until age 20–24 months. Information on clinical features was collected for each episode of AGE [5]. Earlier studies indicated that rotavirus vaccination did not have any affect on the occurrence or severity of NoV AGE [1, 6]. Therefore, cases in the vaccine and placebo recipients were pooled for this study. Clinical severity of the AGE episodes was assessed using a 20-point severity score described by Ruuska and Vesikari [7].

NoVs were detected using a reverse-transcription polymerase chain reaction (PCR) assay targeted at polymerase gene region (A) [8, 9]. RNA was extracted from the stool specimens with use of Boom's silica method [10] and transcribed to cDNA with use of SuperScript II RNase H- Reverse Transcriptase (Invitrogen) [1]. The NoV sequences were analyzed by the Food-borne Viruses in Europe Network and aligned to the following EMBL/

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Genbank NoV strains: Grimsby/95/UK (AJ004864), FL408/1996/USA (AF080558), EmmenE006/2002/NL (AB303929), Lanzhou/35666/2002/China (DQ364459), Hilversum/1999 (AF365989), Leeds/90/UK (AJ277608), Melksham/1989/UK (X81879), Hawaii/1971/US (U07611), Hesse3/1997/DE (AF093797), Valetta/1995/MT (AJ277616), Birmingham/1993/UK (AJ277612), and Southampton/1991/UK (L07418). NoV GII-4 genotype was then specified using another PCR targeted at the gene encoding for the capsid region (C) [11].

Statistical analyses were performed using the Mann-Whitney *U* test to compare the medians of clinical symptoms (duration of diarrhea and vomiting [days], maximum number of diarrheal stools and vomiting episodes in 24 h, and age months) and SPSS, version 15.0 (SPSS), to analyze the medians of severity score points between NoV GII-4 and the other genotypes. All tests were 2-tailed and were considered to be statistically significant at $P < .05$.

RESULTS

Human caliciviruses were detected in 192 of the 485 episodes of AGE in 405 infants. Of these, 148 cases were associated with NoV and 128 were primary infection due to NoV (in 20 cases, a second NoV infection in the same child was detected during follow-up). Only the primary infections were included in the study material. The yearly observation period was defined from July through June (season). Most (75%) of the cases occurred during the second year of follow-up in 2002 (Table 1).

NoV strains belonging to genogroup GII were found in 110 (86%) of the primary infections, genogroup GI NoV strains were found in 18 cases (14%). Nine different NoV genotypes belonging to either genogroup were detected (GI-2, GI-3, GI-4, GI-6, GIIb, GII-1, GII-2, GII-4, and GII-7). GII-4 genotype was detected in 40 cases (31 %) and the second most common genotype GIIb in 33 cases (26%). GII-4 cases occurred during both epidemic seasons 2001 and 2002 (Table 1). There were no recombinants between GII-4 and other genotypes, as determined from the gene sequences of the polymerase and capsid regions.

The median age of the children was 17 months (interquartile range, 10–19 months). Children infected with norovirus GII-4 were slightly older than children infected with other norovirus genotypes (Table 2), but the age distribution between compared groups was not statistically significant. Clinically, GII-4 was associated with more severe AGE episodes than other NoV genotypes (Table 2). Infants with GII-4 had a longer duration of diarrhea ($P = .006$) and a greater number of diarrheal stools during a 24-h period ($P = .003$) than did those infected with other NoV genotypes. The duration of vomiting in children with GII-4 was longer than in children with infection due to other genotypes ($P = .014$). The overall severity score was also higher in the GII-4–infected cases than in the other cases ($P = .002$).

Table 1. Norovirus (NoV) Genotypes Identified During the 2 NoV Epidemic Seasons 2001 and 2002 in Infants With the First Infection of NoV Gastroenteritis

	Season 1	Season 2	Both years combined
Genotypes	No. (%)	No. (%)	No. (%)
GII-4	7 (22%)	33 (34%)	40 (31%)
GIIb	9 (28%)	24 (25%)	33 (26%)
Other genotypes combined:	16 (50%)	39 (41%)	55 (43%)
GII-1	3 (9%)	2 (2%)	5 (4%)
GII-2	1 (3%)	12 (13%)	13 (10%)
GII-7	8 (25%)	11 (12%)	19 (15%)
GI-2	1 (3%)	1 (1%)	2 (1%)
GI-3	1 (3%)	5 (5%)	6 (5%)
GI-4	2 (6%)	2 (2%)	4 (3%)
GI-6	0 (0%)	6 (5%)	6 (5%)
	32 (25%)	96 (75%)	128 (100%)

DISCUSSION

In outbreaks, GII-4 NoVs have been associated not only with higher attack rate [3] but also with more severe clinical presentation than other NoV genotypes [12]. To our knowledge, this is the first study to examine the severity of primary infection with NoV GII-4 in young children relative to other NoV genotypes. The advantage of studies in young children, compared with adults, is that the severity of episodes can be examined without the effect of any pre-existing immunity. Our results suggest that GII-4 caused more severe disease than other NoV genotypes in primary infections of AGE in young children.

Table 2. Clinical Features of Acute Gastroenteritis in Young Children Associated With GII-4 and Other NoV Genotypes

	Children with NoV genotype		
Variable	GII-4 (no.= 40)	Others (no.= 88)	*P* value
Age, months	18 (16-20)*	17 (9-19)	0.094
Diarrhoea			
Days	2 (1–3.75)*	1 (0-2)	**0.006**
Maximum number of times/day	4 (1- 6)*	3 (0-4)	**0.003**
Vomiting			
Days	2 (1–2.75)*	1 (1-2)	**0.014**
Maximum number of times/day	3 (2–5.75)*	3 (1-5)	0.326
Severity score			
Points	7 (6-9)*,**	6 (4-7)	**0.002**

NOTE. *Reported as median value followed by the interquartile range in parentheses (Mann-Whitney *U*-test).

** The severity score points was assessed according to the clinical symptoms (duration of diarrhoea and vomiting (days), maximum number of diarrhoeal stools and vomiting episodes in 24 hours, fever, treatment and dehydration) [7].

We assumed that the primary infection of AGE detected in an infant represents a primary NoV infection in the majority of children. We could not confirm this, because we did not have serum samples for testing pre-existing NoV antibodies. A second infection of NoV AGE was found in only 14% of the children during the 2-year follow-up period. Therefore, it is reasonable to assume that most of AGE episodes detected during the follow-up represented primary NoV infection in these infants.

The next most common genotype after GII-4 was GIIb (26%), as has been reported in outbreaks in adults and older children in Sweden and Finland [13, 14]. In this study, GIIb did not cause more severe infection than the remaining NoV genotypes. We found more cases of NoV AGE during 2002 than during 2001. This may simply reflect the greater likelihood of children to experience NoV AGE in the second than in the first year of life. Alternatively, new GII-4 variants appeared to cause an unusual epidemic peak of outbreaks during 2002 in Europe [3], and such activity might also be associated with the greater number of cases in children during 2002.

In conclusion, the finding of greater clinical severity of the first in lifetime NoV AGE episodes associated with GII-4 NoV, compared with other NoV genotypes, suggests that GII-4 has greater inherent virulence than other NoV types. However, the molecular basis of such enhanced virulence remains speculative and requires further study.

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Genetic analyses of norovirus GII.4 variants in Finnish children from 1998 to 2013

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ABSTRACT

Noroviruses (NoVs) are the major causative agents of acute gastroenteritis (AGE) in outbreaks and in sporadic AGE in young children. Since the mid-1990s, NoV genotype GII.4 has been predominant worldwide. New GII.4 variants appear every two to three years, and antigenic variation is focused on the highly variable protruding domain (P2) of the NoV capsid protein which contains the receptor-binding regions.

We studied NoV GII.4 variants in cases of endemic AGE in Finnish children from 1998 to 2013. Fecal specimens were collected from cases of AGE followed prospectively in rotavirus vaccine trials from 1998 to 2007, and from children seen at Tampere University Hospital because of AGE from 2006 to 2013. Partial capsid sequences were identified with RT-PCR and sequenced allowing P2 domain alignment and phylogenetic comparison of different GII.4 strains, with virus-like particles (VLPs) developed as candidate vaccines.

Of 1495 NoV positive specimens 829 (55%) were of the GII.4 genotype, and altogether twelve GII.4 variants were identified. Identical GII.4 variants were detected in outbreaks of NoVs worldwide. A phylogenetic tree of the amino acid changes in the P2 region showed nine variants that arose over time.

Our data indicates that GII.4 continues to be the predominant NoV genotype circulating in the Finnish community, and the changes in the P2 domain over time result in the development of new variants that cause AGE in children. Future NoV vaccines should either induce type specific immunity for each variant or, alternatively, induce broadly reactive protective immunity covering multiple variants.

1. Introduction

Noroviruses (NoVs) cause outbreaks of acute gastroenteritis (AGE) in all age groups and also sporadic or endemic AGE in children (Glass et al., 2009). In hospital-based and community-based studies NoVs have been found to be the second most common cause, after rotaviruses, of seasonal AGE in children (Pang et al., 1999, 2000; Puustinen et al., 2012; Rasanen et al., 2011). More recently, since the introduction of a rotavirus vaccine into the National Immunization Programme in Finland in 2009, NoVs have become the leading cause of AGE in children in Finland (Hemming et al., 2013) as well as in the US (Payne et al., 2013). In developing countries, NoVs cause an estimate 900,000 episodes of gastroenteritis in children under five years of age, and result in more than 200,000 deaths each year (Patel et al., 2008).

The NoV genome consists of three open reading frames (ORFs). ORF1 encodes non-structural proteins, e.g. RNA-dependent RNA polymerase (RdRp) whereas ORF2 encodes the major structural capsid protein (VP1), and ORF3 encodes the minor capsid protein (VP2). The major protein VP1 contains a conserved shell (S) domain and a protruding (P) domain which is divided into the highly variable P2 domain and the more conserved P1 domain (Prasad et al., 1999). Expression of the major capsid protein VP1 in recombinant baculovirus results in the formation of virus-like particles (VLPs) (Jiang et al., 1992), and NoVs VLPs have been proposed as a candidate NoV vaccine (Ball et al., 1999).

The classification of NoVs is based on the differentiation of the nucleotide sequence. NoVs infecting humans are divided into two main genogroups, GI and GII, and there are at least nine GI and 21 GII genotypes (Green, 2013). The amino acid sequence similarity of the NoV GII capsid protein VP1 need to be more than 85% within the same genotype (Kroneman et al., 2013). NoV GII.4 has been the predominant genotype to emerge worldwide, with new variants over the last twenty years (Lindesmith et al., 2008). Between 1995 and 2006 at least four major pandemics caused by GII.4 variants have been identified. The first pandemic was caused by US95/96 in the mid-1990s (Noel et al., 1999), and it was

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followed by Farmington Hills 2002 (Lopman et al., 2004), Hunter 2004 (Kroneman et al., 2006), Yerseke 2006a and Den Haag 2006b (Tu et al., 2008). Since 2006, three novel variants have appeared: Apeldoorn 2008, New Orleans 2010 (Pang et al., 2010; Siebenga et al., 2010; Vega et al., 2011) and Sydney 2012 (van Beek et al., 2013).

Most genetic variations in VP1 occur in the highly variable P2 domain (Lindesmith et al., 2012a,b, 2013), which binds to the polymorphic histo-blood group antigens (HBGAs), the putative receptors for NoV entry, and interacts with neutralizing antibodies (Cao et al., 2007; Harrington et al., 2002; Lindesmith et al., 2008, 2012a,b; Tan et al., 2003). Antibodies which block interaction of NoV VLPs with these HBGAs are considered indicative of protective immunity (Harrington et al., 2002; Lindesmith et al., 2008, 2011; Nurminen et al., 2011; Reeck et al., 2010). Five common antigenic sites (epitopes A–E) at P2 domain have been identified as blockade epitopes mapped on the surface of the capsid protein (Lindesmith et al., 2012a,b, 2013). The amino acid (aa) changes in these epitopes, especially A, D and E, have been described as a driving force in the evolution of GII.4 variants (Lindesmith et al., 2008, 2012a,b, 2013). Epitope A (aa 294, 296–298, 368, 372) is a conformational epitope close to the HBGA binding pocket (Allen et al., 2008; Debbink et al., 2012; Lindesmith et al., 2008). Epitope D (aa 393–395) was first recognized as a secondary HBGA binding site (Allen et al., 2008; Cao et al., 2007; Lindesmith et al., 2012a,b). Epitope E consists of aa 407, 412, and 413, and has been shown to be strain specific (Lindesmith et al., 2012a,b).

Here we describe the occurrence of twelve NoV GII.4 variants in AGE cases seen in the Finnish community and in hospitalized Finnish children between 1998 and 2013. We have identified the specific blockade epitopes of the P2 domains of the NoV capsid sequences for each variant. In addition, we compared the sequences and their phylogenetic alignment to those of VLPs that were developed as a candidate NoV vaccine in our laboratory at the Vaccine Researcher Center, University of Tampere, Finland (Blazevic et al., 2011; Huhti et al., 2010).

2. Materials and methods

2.1. Clinical specimens

The specimens were collected from AGE cases of young children (under three years of age) participating in six rotavirus vaccine efficacy trials in Finland from 1998 to 2007 (Vesikari et al., 2004, 2006a,b,c, 2010). The children received either placebo or a rotavirus vaccine. The vaccine study protocols and consent forms had been approved by the appropriate ethics committees and patients or their legal guardians gave their informed consent for participation in the study. Additional stool samples from 2006 to 2008 (Rasanen et al., 2011) and from 2009 to 2011 (Hemming et al., 2013) were collected from children (under 16 years of age) seen either in the emergency room or admitted to hospital with AGE and the most recent specimens were collected from a prospective study conducted between September 2012 and August 2013 at Tampere University Hospital. As described above these patients were either diagnosed with gastroenteritis when admitted to the hospital or had gastroenteritis symptoms (Hemming et al., 2013; Rasanen et al., 2011). Stool specimens were stored at −20 °C until testing.

2.2. RNA extraction

Viral RNAs were extracted using the QIAamp® Viral RNA Mini Kit (Qiagen, Hilden Germany) according to the manufacturer's instructions. The viral RNAs were extracted from the stool samples collected in 2000–2002 using a silica binding assay as described previously (Boom et al., 1990). Aliquots of RNA extracts were stored at −70 °C until testing by RT-PCR.

2.3. RNA polymerase (region A) reverse transcriptase (RT)-PCR

Our laboratory has previously identified NoV genotypes, including those presented in this study using reverse transcriptase (RT)-PCR for region A (Hemming et al., 2013; Puustinen et al., 2012; Rasanen et al., 2011). The samples were tested for the presence of human caliciviruses using the primer mixture p289H,I,IUB (antisense) and p290H,I,J,K,IUB (sense) with the RT-PCR method described earlier (Farkas et al., 2004; Jiang et al., 1999), with a slight modification (Puustinen et al., 2012). This RT-PCR detects the RNA polymerase region, amplifying a 319 bp amplicon for NoVs and 331 bp amplicon for sapoviruses. In this study, the RT-PCR for region A was used only to detect the presence of NoVs. The amplicons were stored at −20 °C for sequencing.

2.4. Capsid (region C) RT-PCR

To confirm the NoV GII.4 genotype identified by the RT-PCR for region A described above and to detect the NoV GII.4 variants in each year, all NoV GII.4 specimens in this study were amplified using JV21 (antisense), and JV24 and JV24mod (sense) primers in the capsid (region C) RT-PCR (Buesa et al., 2002; Puustinen et al., 2012). Viral RNAs were first reverse-transcribed using Superscript™ reverse transcriptase (Invitrogen, Carlsbad, CA) as described earlier (Pang et al., 2005; Puustinen et al., 2012). A 317-bp fragment from the beginning of the NoVs' capsid region in ORF2 was amplified using GoTaq® polymerase (Promega, Madison, WI, USA) as described earlier (Buesa et al., 2002; Puustinen et al., 2012). The amplicons were stored at −20 °C for sequencing.

2.5. Capsid (region P) RT-PCR

The most common GII.4 variant in each season was chosen for P region RT-PCR testing. The capsid region P2 of the ORF2 was amplified using the primers ORF1/2-F1 (sense) and P2R2 (antisense), amplifying the N-terminus and P2domain of the capsid region (Allen et al., 2008). Extracted RNA was first diluted 1/20 in aqua sterilisata (Fresenius Kabi). Five microliters of diluted RNA sample was then incubated with 1 µl of 25 µM P2R2-reverse primer at 65 °C for 5 min. The RT-reaction mix contained 1× first strand buffer, 0.5 mM dNTP, 0.01 M DTT, 40 U RNase OUT™ and 200 U SuperScript® III reverse transcriptase (Invitrogen). The RT-PCR reaction conditions were 25 °C for 5 min, 50 °C for 50 min, and 70 °C for 15 min.

The PCR reaction mix contained 0.5× GoTaq® Flexi buffer, 1 mM $MgCl_2$, 0.2 mM dNTP, 0.5 µM ORF1/2-F1 forward primer, and 2 U GoTaq® DNA Polymerase (Promega) in a volume of 30 µl. The 35-cycle PCR was run with the following conditions: primary denaturation at 94 °C for 2 min, denaturation at 94 °C for 45 s, annealing at 58 °C for 45 s, extension at 72 °C for 1 min 40 s and final extension at 72 °C for 10 min.

2.6. The production of VLP

NoV GII.4 (GenBank sequence database accession number AF080551) was isolated from a patient's stool in Finland in 1999. RNA was extracted from the stool with an RNA isolation kit (Qiagen, Hilden, Germany). A 1.6 kb DNA fragment containing the complete gene of the NoV capsid was amplified. The NoV ORF2 capsid gene was cloned to a pCR2.1-TOPO-vector and further subcloned into a baculovirus pFastBac1 donor plasmid (Koho et al., 2012). VLPs were produced using the Bac-to-Bac® baculovirus expression

system in Sf9 insect cells according to the manufacturer's instructions (Invitrogen). VLPs were purified with a discontinuous sucrose density gradient as we have earlier described (Huhti et al., 2010). The AF080551 VLP is being considered for use as a vaccine candidate (Blazevic et al., 2011; Huhti et al., 2010).

2.7. Sequencing and genotyping

All NoV positive PCR products were sequenced with the method described earlier (Puustinen et al., 2012) except for the P2F and P2R2 primers used for sequencing the P2 domain (Allen et al., 2008). Amplicons were purified with the Qiaquick® Gel Extraction Kit (Qiagen) and sequenced using the Big Dye® Terminator v.1.1 Cycle Sequencing Kit (Applied Biosystems). Sequences were obtained using the ABI PRISM® 310 Genetic Analyzer. The sequences were analyzed with Sequencher™ 4.8 software (Gene Codes Corp., USA). The genotyping and the characterization of NoV GII.4 variants were done using the Food-borne Viruses in Europe (FBVE) network www.rivm.nl/mpf/norovirus/typingtool and GenBank http://www.ncbi.nlm.nih.gov/Genbank/index.html. The accession numbers according to NoV GII.4 capsid variants used in this study are the following: Camberwell 1994 AF145896; US95/96 AJ004864; AF080551; AB303929; Farmington Hills 2002 AY485642; Asia 2003 AB220921; Hunter 2004 AY883096; Terneuzen 2006a EF126964; Den Haag 2006b EF126965; AB434770; Apeldoorn AB445395; New Orleans 2010 GU445325; Sydney 2012 JX459908. When analyzing the variants within GII.4, the accession numbers and the variants' names from FBVE were used for sequence nomenclature. However, the final analysis (described below) was performed from the original strains extracted from the AGE children, not from the reference strains.

2.8. Alignment and phylogenetic analyses

The nine most common amino acid sequences of the P2 domains from NoV GII.4 AGE cases from 1998 to 2013 and the previously identified P2 domain of the VLP (AF080551) (Blazevic et al., 2011; Huhti et al., 2010) were aligned using Clustal X (version 1.8) and GeneDoc (version 2.7.000). A phylogenetic tree was generated using the neighbor-joining method by comparison of the P2 amino acid sequences (143 aa, nucleotide position of the P2 domain in NoV genome 5922–6300) obtained for ten NoV GII.4 strains and the NoV GII.3 strain using MEGA 3.1 software (Kumar et al., 2004) with a bootstrap value of 1000. The amino acid sequence of the P2 domain of NoV GII.3 (accession number EU187437) from GenBank was used as the standard (non-GII.4). Evolutionary distances were calculated using the Poisson correction method.

3. Results

3.1. Prevalence of NoV GII.4 in Finnish children between 1998 and 2013

In the present study, we determined NoV GII.4 variants according to capsid region in these peak years (1998–1999, 2002–2003 and 2006–2007) as well as the years between these peaks. In the community-based study, NoV was identified as the causative agent in 1,071 cases of AGE during the study period of 1998–2007 (Table 1). Of these, 494 (46%) cases were caused by NoV GII.4. Distribution of GII.4 genotypes varied from being 17% in the 2003–2004 season and reaching 98% in the peak season of 2006–2007 (Table 1). In addition, in five NoV seasons from August 2006 to June 2013, 424 NoV positive-specimens were collected from children that were seen at Tampere University Hospital because of AGE. NoV GII.4 was the most common genotype in those children; it was detected in 335 (79%) of cases (Table 2). The prevalence of NoV GII.4 and other NoV genotypes in sporadic AGE cases are shown in Fig. 1. Altogether, NoV was detected in 1,495 of non-outbreak cases of AGE in children between 1998 and 2013; of these 829 (55%) were of the GII.4 genotype.

3.2. Epidemic years of NoV GII.4 variants

Within the study period, twelve GII.4 variants were detected in Finland (Tables 1 and 2). During the 1998–1999 season, the US95/96 variant was identified and was the only GII.4 variant associated with the epidemic during this period. From 1999 to 2002, a newly circulating variant, Farmington Hills 2002, was identified in the epidemic season of 2002–2003. However, a few cases of the US95/96 variant were also seen during this new epidemic season. The emergence of new GII.4 variants Terneuzen 2006a and Den Haag 2006b apparently resulted in the most severe epidemic season during this study from 2006 to 2007. These variants were equally represented in community-based AGE cases (Table 1), whereas the Den Haag 2006b was more common than Terneuzen 2006a in children seen at hospital (Table 2). Interestingly, the

Table 1
NoV GII.4 variants in sporadic cases of acute gastroenteritis in Finnish children in the community between 1998 and 2007.

Season[a]	**1998–1999**	1999–2000	2000–2001	2001–2002	**2002–2003**	2003–2004	**2006–2007**	Total (%)
NoV GII.4	**213 (72%)**[b]	57 (19%)[c]	68 (37%)[d]	49 (32%)	**48 (86%)**	3 (17%)	**56 (98%)**	494 (46%)
GII.4 subtypes								
Camberwell_1994		1						
US95/96_1996	76	28	23	20	9			
2001			1	10				
Farmington Hills_2002			1	19	39	1	1	
Asia_2003							1	
Hunter_2004						2		
Terneuzen_2006a							26	
Den Haag_2006b							27	
2007							1	
N[e]	294	308	186	152	56	18	57	1071

Bold values indicate peak year of GII.4.
Nov GII.4 variants were defined according to capsid region C.
[a] Season is a time period from July to June.
[b] 76 (36%) cases of NoV GII.4 positive-specimens were randomly selected for GII.4 variant identification.
[c] 29 (51%) cases of NoV GII.4 positive-specimens were randomly selected for GII.4 variant identification.
[d] 25 (37%) cases of NoV GII.4 positive-specimens were randomly selected for GII.4 variant identification.
[e] Total number of NoV-positive specimens.

Table 2
NoV GII.4 variants in cases of acute gastroenteritis in children seen at Tampere University Hospital in five seasons between 2006 and 2013.

Season[a]	2006–2007	2007–2008	2009–2010	2010–2011	2012–2013	Total (%)
NoV GII.4	138 (95%)	78 (76%)	37 (74%)	36 (59%)	46 (71%)	335 (79%)
GII.4 subtypes						
Hunter_2004	5					
Terneuzen_2006a	46	1				
Den Haag_2006b	87	77			8	
2007			1			
Apeldoorn_2008			3			
New Orleans_2010			33	36	9	
Sydney_2012					29	
N[b]	146	102	50	61	65	424

Nov GII.4 variants were defined according to capsid region C.
[a] Season is a time period from July to June.
[b] Total number of NoV-positive specimens.

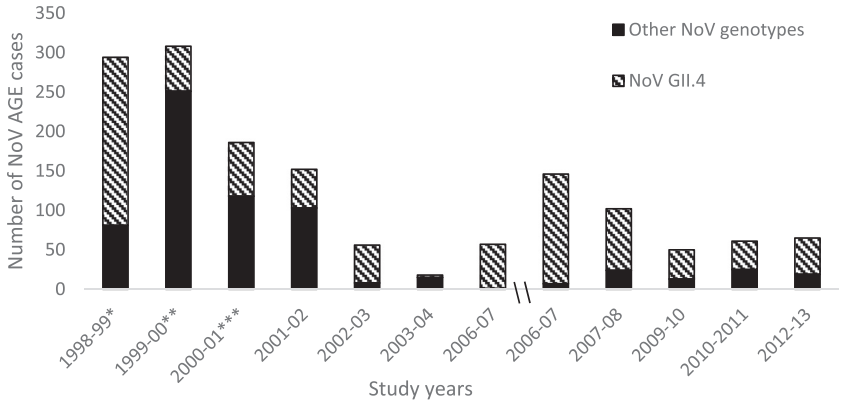

Fig. 1. The distribution of NoV GII.4 in Finnish children throughout the 1998–2013 study period. Two lines separate the sporadic and hospitalization NoV AGE cases. *76 (36%) cases of NoV GII.4 positive-specimens were randomly selected for GII.4 variant identification. **29 (51%) cases of NoV GII.4 positive-specimens were randomly selected for GII.4 variant identification. ***25 (37%) cases of NoV GII.4 positive-specimens were randomly selected for GII.4 variant identification.

Den Haag 2006b caused almost three times as many NoV cases as the Terneuzen 2006a variant in young children: 199 and 73 cases, respectively. Apeldoorn 2008 was seen only occasionally in the study season 2009–2010. The New Orleans 2010 variant predominated during the two seasons between 2009 and 2011, and a few cases were also seen in the 2012–2013 epidemic season. In the 2012–2013 season a variant Sydney 2012, occurred causing 60% of all NoV GII.4 cases. Altogether in this study, we found that six GII.4 variants (US95/96, Farmington Hills 2002, Terneuzen 2006a, Den Haag 2006b, New Orleans 2010 and Sydney 2012) were associated with most cases of AGE in children.

3.3. Alignment of the P domain of NoV GII.4 variants

Fig. 2 shows the amino acid alignment and comparison of the P2 domain capsid sequences of GII.4 variants in Finland from 1998 to 2013, including the corresponding sequences from the VLP candidate vaccine. The amino acid sequence of the GII.4 VLP was included in this study to compare its antigenic similarity with various GII.4 variants. NoV GII.4 blockade epitopes (A–E) in the P2 domain are also shown in Fig. 2. We compared the changes in the blockade epitopes of different GII.4 strains found in sporadic NoV GII.4 AGE cases in Finnish children to the reference strain (AF080551 VLP) (Fig. 2) and found that a change in position 294 of epitope A only occurred in the 2008, 2010, and 2012 strains (aa replacement 294A, 294T, 294P, 294T). Five variable aa at positions 296–298, 368, and 372 were identical in the 2008 and 2010 strains, whereas a change at position 368 occurred in the 2012 strain. Epitopes B–D were similar in the 2004 and 2006a strains and only two aa changes occurred, at positions 298 and 407. In our material, epitope E seemed to vary in every epidemic strain. The 2010 GII.4 strain had several aa substitutions in all blockade epitopes compared to previously circulating variants. The changes in aa sequences of the blockade epitopes between precursor GII.4 variant AF080551 and more recent GII.4 variants appeared to follow no particular direction, and the previously occurring changes rarely reappeared.

3.4. Phylogenetic analysis of NoV GII.4 variants

A phylogenetic tree was generated from the alignment of the P2 domain of ten GII.4 amino acid sequences (143 aa), including VLP sequences (AF080551) (Fig. 3). The tree shows nine NoV GII.4 variants that arose over time, starting from variant US95/96 through the variant Sydney 2012. The GII.4 variants associate with the epidemics shown in Tables 1 and 2. Most of the variants seem to have evolved from the previously circulating variants. Surprisingly, variants Terneuzen 2006a and Den Haag 2006b aligned into different sublineages. Den Haag 2006b was linked to the variants that previously circulated in Finland Apeldoorn 2008, New Orleans 2010 and Sydney 2012 whereas the variant Terneuzen 2006a associated closely with the variants Hunter 2004 and Farmington Hills 2002. The new Sydney 2012 variant was linked with common ancestors in the variants Apeldoorn 2008 and New Orleans 2010.

Strains	Epitopes																
	A						B		C		D			E			
	294	296	297	298	368	372	333	382	340	376	393	394	395	407	412	413	
GII.4 VLP	A	S	H	D	T	N	L	K	E	Q	N	–	N	N	T	G	
US95/96 1996							V				S	–					
GII.4 2001			R	N	S	D			S		D	–	R	D			
Farmington Hills 2002		T		N	N		M		G	E		G	T	S			
Hunter 2004		T	Q	N	S	S	V	R	R	E	S	T	T	D	D	S	
Terneuzen 2006a		T	Q	E	S	S	V	R	R	E	S	T	T		D	S	
Den Haag 2006b			R	N	S	E	V		G	E	G	T	T	S	N	V	
Apeldoorn 2008	T		R	N	A	D	V		A	D	D	T	A	S	N	S	
NewOrleans 2010	P		R	N	A	D	V		T	E	S	T	T	S	N	I	
Sydney 2012	T		R	N	E	D	V		T	E	S	T	T	S	N	T	

Fig. 2. Comparison of amino acid sequences of blockade epitopes of the norovirus GII.4 strains. The most common GII.4 variants found in community cases and hospitalized children were compared. The sequence from GII.4 VLP (AF080551) is used as a reference sequence. The nomenclature of GII.4 strains presented here was made according to FBVE. However, these strains were defined from NoV GII.4 AGE children between 1998 and 2013. The amino acids different from the reference strain are marked. Amino acid numbering is indicated at the top. Epitope A; gray, epitope B; green, epitope C; blue, epitope D; yellow and epitope E; pink (Lindesmith et al., 2012a,b). The GII.4 variant used for VLP production is shown in orange.

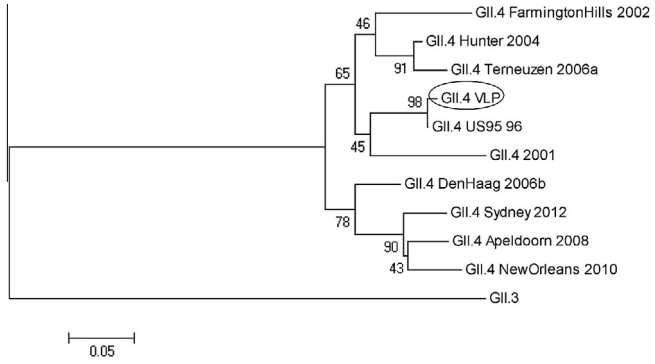

Fig. 3. Phylogenetic analysis of amino acid sequences of the P2 domain (143 aa) from norovirus GII.4 strains circulating in Finnish children between 1998 and 2013. The nomenclature of GII.4 strains presented here was made according to FBVE. GII.4 VLP (AF080551) is circled. The amino acid sequence of the P2 domain of NoV GII.3 (accession number: EU187437) was used as the standard (non-GII.4). The scale bar represents the number of the amino acid substitutions per site.

4. Discussion

Previously, we have shown that the epidemic years of NoV AGE in children occurred together with an increase of NoV GII.4 (Puustinen et al., 2012). Here we show the prevalence of NoV GII.4 variants in eleven seasons of AGE in children in Finland from 1998 to 2013. Since the highly variable P2 domain has been suggested to contain the most important antigenic changes of the capsid region (Lindesmith et al., 2012a,b, 2013). We investigated the genetic changes of the P2 domain in the NoV capsid region in these variants. Blocking assays using sera from immunized mice and from outbreaks in humans suggests that antigenic variation maintains GII.4 persistence in the face of human herd immunity (Lindesmith et al., 2012a,b, 2013).

The new GII.4 variants rapidly spread around the world; as our results show, the epidemic years of AGE in children are always associated with novel GII.4 variants. During this study, seven different GII.4 variants (US95/96, Farmington Hills 2002, Hunter 2004, Terneuzen 2006a, Den Haag 2006b, New Orleans 2010 and Sydney 2012), were clearly associated with epidemic years in non-outbreak AGE cases in children, similar to what others have reported for NoV outbreaks (Lopman et al., 2004; Pang et al., 2010; Siebenga et al., 2007; Vega et al., 2011). The first GII.4 variant, US95/96, was recognized in 1995, causing epidemics in the US (Noel et al., 1999) and in Europe (Lopman et al., 2002). In Finland, the US95/96 variant occurred from 1998 to 1999, causing an epidemic peak in children. The spread of a new GII.4 variant, Farmington Hills 2002 caused epidemics and an atypically large number of outbreaks during the summer and autumn months in Europe (Lopman et al., 2002); the same was also seen in sporadic AGE cases in our study. Siebenga et al. (2009) reported that the Hunter 2004 variant also caused epidemics (Siebenga et al., 2009). However, this variant was rare in our study material and is not associated with any epidemic year. Farmington Hills 2002 was then replaced by two-circulating GII.4 variants (Terneuzen 2006a and Den Haag 2006b) in early 2006. The Den Haag 2006b was also distributed globally (Siebenga et al., 2009). A high prevalence of NoV cases was observed in Finnish children in connection to these new variants, as compared to previous epidemic years. Pang et al. (2010) reported that the Den Haag 2006b caused significantly more outbreaks than the Terneuzen 2006a in Alberta, Canada (Pang et al., 2010). We also show that the Den Haag 2006b was more common than the Terneuzen 2006a in NoV AGE cases in children seen at hospital. A new variant, New Orleans 2010, was first detected in October 2009 in the USA (Vega et al., 2011) and we identified the first cases already in December 2009 (Puustinen et al., 2011). While this novel variant was the most common one in children seen at hospital, it did not cause such a widespread epidemic as the 2006 variants. Sydney 2012, the most recent, variant evolved from previous variants Apeldoorn 2008 and New Orleans 2010 but it is phylogenetically distinct (van Beek et al., 2013). Altogether, variants found in this study included epidemiologically significant strains that caused epidemics and outbreaks across the world.

The present findings of phylogenetic analyses of the P2 domain in sporadic AGE cases are comparable with earlier findings in outbreaks. Surprisingly, two different variants (Terneuzen 2006a and Den Haag 2006b) emerged simultaneously worldwide in the same year. However, the variants are considerably different. Consistent with the observation from outbreaks in Canada, Europe (Pang et al., 2010; Siebenga et al., 2009), and in pediatric AGE cases in Finland, the Terneuzen 2006a variant was closely linked to the Hunter 2004 variant in pediatric AGE cases in Finland. The Den Haag 2006b variant, on the other hand, was associated with the variants Apeldoorn 2008 and New Orleans 201, as shown previously (Pang et al., 2010; Siebenga et al., 2009; Vega et al., 2011). Our assumption is that in the Den Haag 2006b variant a significant number of amino acid changes have led to better host

immunity evasion, which is why it occurred more frequently and caused more severe disease in children than the Terneuzen 2006a variant.

As previously reported in studies of outbreaks, in this study we detected the same amino acids changes in specific VP1 sites in sporadic AGE cases in children (Lindesmith et al., 2012a,b, 2013). Studies of human and mouse anti-NoV antibodies, together with molecular biology approaches, have identified three significant blockade epitopes (A, D, and E) between the GII.4 strain that circulated from 1987 to 2012 (Debbink et al., 2012, 2013; Lindesmith et al., 2012a,b). The widespread NoV epidemics associated with the Farmington Hills 2002 followed dramatic changes in the form of amino acid insertions (Lindesmith et al., 2012a,b; Lopman et al., 2004). We also show the changes in these epitopes which seem to vary in every pandemic strain, unlike in epitopes B and C. Epitope A differs between strains and may be an effective predictor of an emergence of a new NoV strain (Lindesmith et al., 2013). Epitope D has been shown to modulate the HBGA binding of the GII.4 strain (Lindesmith et al., 2008). In addition, epitope E has been detected to be a specific neutralizing blockade epitope for the Farmington Hills 2002 (Lindesmith et al., 2012a,b). However, the changes at these sites do not reappear in novel emerging variants. It is therefore extremely challenging to predict the next changes to appear in novel GII.4 strains. We also included in this study the amino acid sequences from NoV GII.4-1999 AF080551 VLPs used as a candidate NoV vaccine developed by our laboratory (Blazevic et al., 2011; Huhti et al., 2010). We showed that mice immunized with NoV GII.4-1999 VLP developed cross-reactive blocking antibodies to the more recent NoV GII.4 VLP New Orleans 2010 (GU445325) and Sydney 2012 (JX459908) variants (Blazevic et al., 2011; Tamminen et al., 2012, 2013, and unpublished observation). Even though the amino acids in common blockade epitopes A–E are highly variable in the GII.4 VLP-1999, GII.4 VLP-2010, and GII.4 VLP-2012 (Fig. 2) the blocking antibody response we observed indicates cross-protective immunity. Monoclonal antibodies (mAbs) to GII.4 VLPs-1987 blocked the strains circulating up to 2002 (Lindesmith et al., 2011). On the other hand, GII.4-1987 polyclonal mouse sera did not block H type 3 interaction with GII.4-2006 (Debbink et al., 2012). Our results indicate that there are still unknown epitopes that have a role in the NoV blockade, as has been proposed earlier (Lindesmith et al., 2012a,b). The fast antigenic variation of the capsid region P2 complicates NoV vaccine design. NoV VLPs vaccine needs to be frequently reformulated, or, as we suggest, high levels of cross-blocking antibodies may be sufficient for cross-protection across different variants. Currently, the GII.4 consensus VLP vaccine, which includes three genetically different GII.4 strains, is under clinical trials (Parra et al., 2012).

In conclusion, the NoV GII.4 variants that emerged worldwide were also associated with epidemic years of AGE in children in Finland. NoV GII.4 strains undergo antigenic variation by changing amino acids in the P2 domain between epidemics in response to herd immunity (Lindesmith et al., 2013). This is an indication that immunity induced by natural NoV infection is type-specific and not very long lasting (Lindesmith et al., 2012a,b; Rockx et al., 2005). The antigenic variation must be considered in NoV vaccine design. However, in contrast to natural infection immunity induced by NoV infection in children, VLP vaccines may induce high levels of cross-protective antibodies which may be sufficient for durable protection across variant strains.

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BRIEF REPORT

A comparison of methods for purification and concentration of norovirus GII-4 capsid virus-like particles

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Abstract Noroviruses (NoVs) are one of the leading causes of acute gastroenteritis worldwide. NoV GII-4 VP1 protein was expressed in a recombinant baculovirus system using Sf9 insect cells. Several methods for purification and concentration of virus-like particles (VLPs) were evaluated. Electron microscopy (EM) and histo-blood group antigen (HBGA) binding assays showed that repeated sucrose gradient purification followed by ultrafiltration resulted in intact VLPs with excellent binding to H type 3 antigens. VLPs were stable for at least 12 months at 4°C, and up to 7 days at ambient temperature. These findings indicate that this method yielded stable and high-quality VLPs.

Noroviruses (NoVs) cause sporadic acute nonbacterial gastroenteritis in all age groups [12, 13]. NoVs are divided into five genogroups, GI to GV, of which GI and GII strains mostly affect humans [21]. Recently, NoV genotype GII-4 has been responsible for the majority of sporadic gastroenteritis cases and outbreaks [13]. The NoV genome consists of a single-stranded RNA of about 7.6 kb, organized into open reading frames (ORF 1–3). ORF-1 codes for the RNA-dependent RNA polymerase, and ORF-2 and -3 encode the two structural proteins VP1 and VP2 [10].

Expression of the capsid VP1 gene by recombinant baculoviruses leads to self-assembly into empty virus-like particles (VLPs) that are morphologically and antigenically similar to native NoV [9]. NoV VLPs are widely used as antigens in diagnostic serological assays and as candidate vaccines against NoVs [2, 7]. NoV VLPs are highly stable and resistant to variable conditions, particularly to low pH [1, 9].

There are limitations in NoV VLP production in terms of inadequate yield and quality of the VLPs [1, 3, 9, 20]. Both sucrose and CsCl gradients ultracentrifugation have been used for purification of NoV VLPs [1, 7, 9, 17], even though studies on rotavirus-like particles demonstrated a low yield and impurities resulting from CsCl gradient purification [16].

In the present study, we compared commonly used methods for NoV GII-4 VLP purification [1, 14, 17] and concentration [6, 19], considering the purity, yield, morphological integrity, antigenicity and functionality of the purified VLPs.

The steps for cloning the NoV GII-4 (GenBank sequence database accession number AF080551) full-length capsid gene are described elsewhere [11].

VLPs were produced in Sf9 insect cells infected with the recombinant baculovirus according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Baculovirus titers expressed as the multiplicity of infection (MOI) of the P2 stocks were determined using a BacPak Rapid Titer Kit (Clontech Laboratories, Mountain View, CA).

At day 6, infected cell culture (200 ml) was clarified by centrifugation at 3000×*g* for 30 min at 4°C. VLPs in the supernatant were concentrated by ultracentrifugation (L8-60M ultracentrifuge, Beckman SW-32.1 Ti rotor) at

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100,000×*g* for 2 h at 4°C, and pellets were resuspended in 0.2 M Tris–HCl, pH 7.3. VLPs were loaded onto a 10–60% discontinuous sucrose gradient and ultracentrifugated at 100,000×*g* for 1 h at 4°C as described before [17]. Fractions were collected by bottom puncture. The fractions containing VLPs were pooled. An additional discontinuous sucrose gradient (35–60%) ultracentrifugation was performed. Sucrose was removed by overnight dialysis against 1 liter of PBS. VLPs were concentrated by dialysis against polyethylene glycol (PEG; 50%) [6] or by ultrafiltration [19]. VLPs were concentrated using an Amicon Ultra 30 kDa centrifuge filter device (Millipore Corporation, Billerica, Germany). VLPs were stored at 4°C in PBS.

Alternatively, a less time-consuming sucrose density gradient purification method was employed [14]. Clarified supernatants were pelleted twice by ultracentrifugation. Pellets were resuspended in 0.2 M Tris–HCl, pH 7.3, and placed on a discontinuous sucrose density gradient (10–60%) for ultracentrifugation at 100,000×*g* for 16 h at 4°C. The VLP band, which was visible at the 35% sucrose layer, was collected. Sucrose was removed by dialysis against 1 liter of PBS, and VLPs were concentrated by ultracentrifugation at 100,000×*g* for 2 h at 4°C.

In addition, clarified supernatants were concentrated, and the pellets were resuspended in sterile water. VLPs were sedimented by ultracentrifugation through cesium chloride (CsCl) (0.4 g/ml) at 116,000×*g* for 18 h at 4°C as described earlier by others [1]. CsCl was removed by dialysis against PBS, and VLPs were concentrated using an Amicon Ultrafilter.

The total protein content of the purified VLP preparation was determined using the Pierce BCA Protein Assay (Thermo Science, Rockford, USA). Endotoxin levels in the VLP preparations were quantified using the Limulus amebocyte lysate (LAL) assay (Lonza, Walkersville, MD, USA). The level of endotoxin was <0.1 EU/10 µg of protein, which is below the international standard of ≤30 EU/20 µg of protein [15]. All samples were analyzed for protein expression by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot. The presence of NoV VLPs was verified by electron microscopy (EM). VLP preparations were negatively stained with 3% uranyl acetate (UA), pH 4.6. The VLPs were examined using an FEI Tecnai F12 electron microscope operating at 120 kV.

Binding of GII-4 VLPs to carbohydrate receptors was examined by the histo-blood group antigen (HBGA) binding assay as described by others [18], with slight modifications. Briefly, VLPs were coated at 50 ng/well, and synthetic biotinylated H type 3 and Lewis^b histo-blood group carbohydrates (Lectinity Holdings Inc. Moscow, Russia) were used in serial threefold dilutions, starting from 6 µg/ml. Wells lacking the synthetic carbohydrates were used as a negative control.

The conditions for Sf9 cell infections were optimized in order to obtain a high yield of the VLPs. For recombinant baculovirus P2 stock, an MOI of 1 was found to be optimal, and VLPs were harvested from the supernatant after 6 days of infection. VLPs were further purified by several different procedures, as described in Fig. 1a.

The best yield (2–3 mg/200 ml) was obtained after the purification procedure described in Fig. 1a, panel B. In comparison, the method described in Fig. 1a, panel C, yielded a ten times lower amount of NoV capsid VLPs. The purity of the VLPs obtained by each method was determined by 12% SDS-PAGE and staining of the proteins with PageBlue (Fig. 1b). 1 µg of the total protein was loaded into each lane. Each method resulted in equally pure protein bands corresponding to the size of the NoV capsid. Residual PEG present in the VLPs, concentrated by PEG dialysis, may have interfered with the protein concentration determination, and

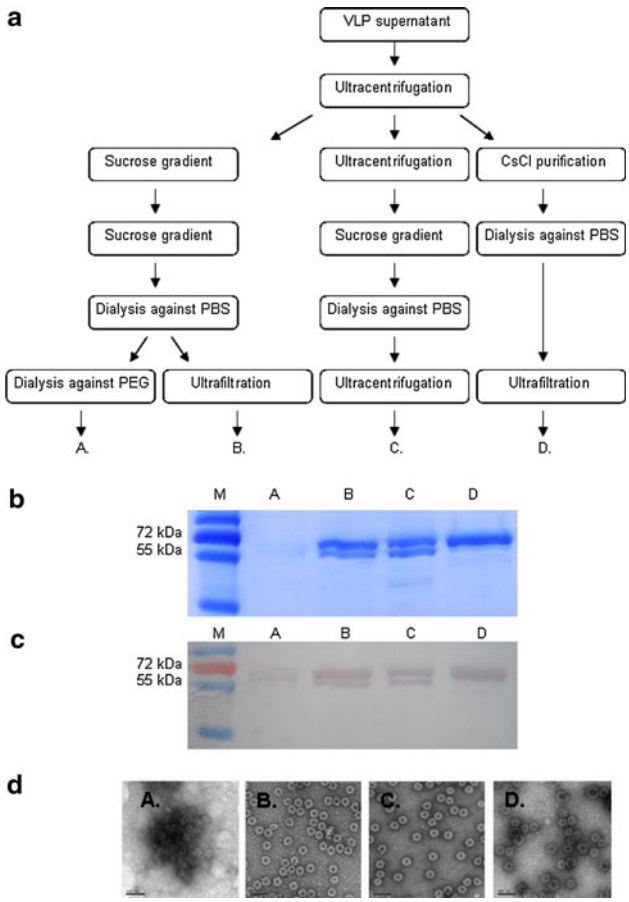

Fig. 1 Purification, concentration and characterization of NoV VLPs. **a** Several purification procedures (*A–D*) were used, and the purity, morphology, antigenicity and yield of the VLPs were compared. **b** NoV capsid protein analysis by 12% SDS-PAGE. **c** Western blot analysis using a human convalescent serum against norovirus GII-4. **d** EM of purified NoV VLPs observed at a magnification of ×30,000. *Bar* 100 nm. Samples from purification procedures *A*, *B*, *C*, and *D* correspond to lanes and panels *A*, *B*, *C*, and *D*, respectively. *M* protein weight marker

this could explain the lower capsid protein concentration observed (Fig. 1b, lane A). NoV capsid identity and antigenicity of the VLPs were assayed by western blot using human NoV-specific convalescent sera (Fig. 1c). The results show pure NoV capsid proteins without degradation and with similar antigenicity for each of the purification methods. Next, we determined the morphological integrity and homogeneity of the VLPs by EM (Fig. 1d). The VLPs obtained by purification with sucrose density gradients followed by dialysis and either ultrafiltration (Fig. 1d, panel B) or ultracentrifugation (Fig. 1d, panel C) were approximately 38 nm in size, with the classical appearance of NoV capsid VLPs. By contrast, CsCl purification (Fig. 1d, panel D) and concentration of the VLPs by dialysis against PEG (Fig. 1d, panel A) resulted in VLPs of heterogeneous size, which appeared broken and aggregated. The VLPs purified and concentrated by the method schematically presented in Fig. 1a, panel B, were of the best quality and were subjected to further analysis.

To determine the optimal storage conditions and stability of the NoV capsid VLPs, VLPs were treated under different conditions, and the samples were analyzed by SDS-PAGE to test their protein integrity (Fig. 2a) and by EM to examine their morphology (Fig. 2b). VLPs were stable for at least 12 months at 4°C in PBS, pH 7.4 (Fig. 2a and b, panel 1), and at room temperature (23°C) up to 7 days (Fig. 2a and b, panel 3). The VLPs withstood the sterile filtration conditions when 0.22-µm filters (Durapore, Millipore, Ireland) were used. Next, we intentionally disrupted the VLP morphology by heat treatment at 60°C for 1 h (Fig. 2b, panel 4) [1] without degrading the capsid protein (Fig. 2a, lane 4).

An HBGA binding assay [8, 18] was used to test the functionality of the purified VLPs as well as to determine the significance of the conformational binding sites on the VLP, which would presumable require intact VLPs. A comparison of the binding of VLPs purified by sucrose gradient centrifugation and by CsCl sedimentation, as well as heat-treated VLPs (60°C, 1 h), to synthetic biotinylated H type 3 carbohydrate is shown in Fig. 3. The binding was clearly dependent on the morphology and preserved structure of the VLPs, with the lowest level of binding observed with the heat-treated VLPs with disrupted conformational binding sites. Lewis^b antigen was used as a control in the assay, and this did not bind to any of the VLPs (Fig. 3).

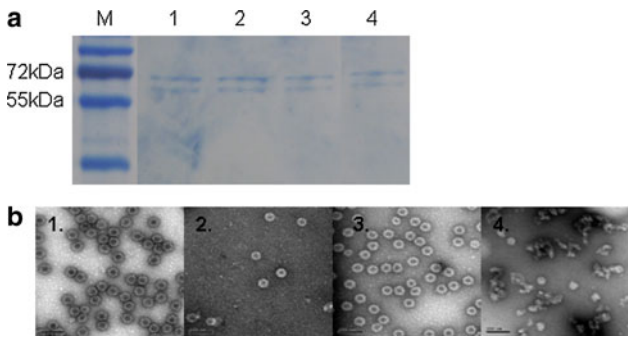

Fig. 2 Stability of NoV VLPs. The stability was analyzed by SDS-PAGE (**a**) and EM (**b**) at a magnification of ×30,000. *Bar* 100 nm. NoV capsid VLPs after 8 months at 4°C (*lane 1 and panel 1*), sterile filtration (*lane 2 and panel 2*), 7 days at room temperature (*lane 3 and panel 3*) and heat treatment (60°C, 1 h) (*lane 4 and panel 4*)

NoV VLPs have been used extensively to study protein interactions [8], and virus assembly [17], and have been used as a tool in diagnostic serological assays [7]. Clinical trials have been performed with the NoV VLPs used as a vaccine [2]. For all these applications, high-quality VLPs would be preferable. In this study, different methods of VLP purification and concentration were used, and the purity, integrity, morphology, antigenicity and functionality of the GII-4 NoV VLP preparations were examined.

VLPs purified by each method (Fig. 1a, methods A–D) had a similar appearance and migration pattern on the SDS-PAGE gel. A western blot with a human convalescent serum from an individual infected with GII-4 confirmed the identity of the capsids and the lack of degradation products.

The reason for the lower protein yield after purification procedure C might have been that a narrow visible band of VLPs was collected from 35% sucrose, causing some of the protein to be omitted, thus affecting the yield. However, a yield of up to 2–3 mg of VLPs, which was obtained by the best purification method described in the present study, is remarkably high when compared to other reports [3, 4].

EM analysis of the morphological integrity and homogeneity of the VLPs showed that VLPs obtained by purification with sucrose density gradients followed by ultrafiltration or ultracentrifugation (procedures B and C,

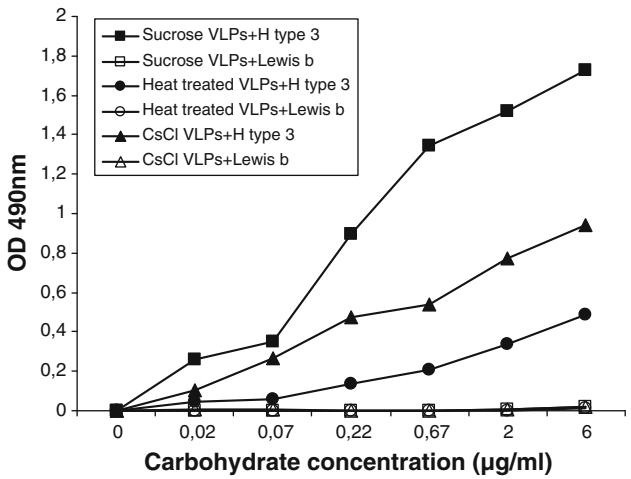

Fig. 3 Binding of NoV VLPs to synthetic ABH histo-blood group antigens. Sucrose-purified VLPs (Sucrose VLPs, purified according to procedure B in Fig. 1a), heat-denatured NoV VLPs (60°C, 1 h) (Heat-treated VLPs) and CsCl-purified VLPs (CsCl VLPs) were tested for binding to H type 3 and Lewis^b carbohydrates at the indicated concentrations. The pH value in the binding assay was 7.4

respectively) were homogenous and intact, and approximately 38 nm in size. However, CsCl-purified VLPs appeared heterogeneous in size, with a few broken particles, although comparable to the morphology seen by others [7]. CsCl purification is known to introduce several impurities at the end of the process and cause aggregation of the VLPs during storage [5]. The poorest morphology was seen after concentration of the VLPs by dialysis against PEG, which resulted in aggregation. In addition, residual PEG, which leaks through the dialysis membrane, might interfere with further applications of the VLPs [19].

Our data clearly demonstrate that the purification process affects the integrity of the native quaternary structure of NoV VLPs and, subsequently, the receptor-binding functionality of the VLPs. Although the majority of the VLPs purified by the CsCl method seemed intact in the EM image, the difference from sucrose-density-gradient-purified VLPs in HBGA binding is striking. This result is supported by the recent finding that CsCl has a negative impact on the functionality of VLPs [5]. We also demonstrated that even heat-disrupted VLPs have binding capability, but intact homogenous VLPs have a significantly greater binding intensity. Standardization of the purification method for NoV VLPs used in diagnostic serological assays and blocking assays [8] would greatly strengthen the results obtained.

To the best of our knowledge, this is the first time that VLPs purified by conventional purification and concentration methods were compared in terms of yield, purity, morphological integrity, antigenicity and functionality. The results show that NoV GII-4 VLPs purified twice by sucrose density gradient centrifugation followed by ultrafiltration maintain their icosahedral capsid structure and their capacity to bind HBGAs.

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ORIGINAL ARTICLE

Characterization and immunogenicity of norovirus capsid-derived virus-like particles purified by anion exchange chromatography

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Abstract Recombinant baculovirus (BV) expression systems are widely applied in the production of viral capsid proteins and virus-like particles (VLPs) for use as immunogens and vaccine candidates. Traditional density gradient purification of VLPs does not enable complete elimination of BV-derived impurities, including live viruses, envelope glycoprotein gp64 and baculoviral DNA. We used an additional purification system based on ionic strength to purify norovirus (NoV) GII-4 capsid-derived VLPs. The anion exchange chromatography purification led to highly purified VLPs free from BV impurities with intact morphology. In addition, highly purified VLPs induced strong NoV-specific antibody responses in BALB/c mice. Here, we describe a method for NoV VLP purification and several methods for determining their purity, including quantitative PCR for BV DNA detection.

Introduction

Baculoviruses (BVs) are dsDNA viruses that naturally infect insects and arthropods and are unable to replicate in mammalian or other vertebrate cells [3, 8, 32]. The recombinant BV expression system in insect cells was developed for protein expression in the 1980s and has since been used in a wide range of applications because many proteins require folding, subunit assembly, and extensive posttranslational modification to be biologically active [18, 54, 57]. This expression system is highly effective in producing large quantities of recombinant viral capsid and envelope proteins that spontaneously assemble into virus-like particles (VLPs) inside the cytoplasm of infected insect cells [18, 33, 42, 54]. These VLPs are morphologically and antigenically similar to native viruses [33] but lack the replicative capacity of the virus, are non-infectious, and do not contain any genetic material. VLPs, moreover, are commonly stable in storage and relatively acid and heat stable [5, 19, 33]. VLPs provide a valuable alternative in the development of diagnostic assays for immunological and epidemiological studies.

Numerous types of VLPs have been produced for various viruses, such as canine parvovirus, Ebola virus, coronavirus, rotavirus, hepatitis B virus and human immunodeficiency virus (HIV) [40, 41, 48, 53, 54, 62]. VLPs have been demonstrated to be highly effective in stimulating adaptive B- and T cell immune responses in mice [19, 37, 62]. Thus, there is considerable potential for using VLPs as vaccine candidates. Indeed, two human papilloma virus (HPV)-based VLP vaccines have been licensed and are currently widely used [49, 51].

Human noroviruses (NoVs) belong to the family *Caliciviridae*. NoVs cause the second most common nonbacterial gastroenteritis after rotavirus in humans of all age groups [21, 38, 44] and are associated with outbreaks of gastroenteritis worldwide [12, 39]. NoVs cause approximately 1 million hospitalizations annually and more than 200,000 deaths worldwide in children under 5 years of age [22, 46]. Most NoVs affecting humans belong to two genogroups (GI and GII), and these two genogroups are divided into at least 8 GI and 17 GII genotypes [66]. Despite this diversity, in recent years, genotype GII-4 has primarily been responsible for the majority of sporadic gastroenteritis cases and outbreaks [39, 56].

NoVs are difficult to study because they do not grow in a cell culture system, and no animal model is available. The

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major capsid protein VP1 is the main target for neutralizing antibodies to NoV [14, 25, 58]. The cloning and expression of the NoV capsid gene VP1 in a BV expression system has been shown to result in the assembly of VLPs that are similar to native virus in size and appearance [33]. NoV VLPs have been used to study protein interactions [25], and virus assembly [50] and is also a source of antigen for diagnostic serological assays and for the development of candidate vaccines against NoVs gastroenteritis [6, 10, 20, 24, 29].

A major challenge for vaccine development is purification of VLPs from recombinant BVs [27, 52]. VLPs used for research purposes are commonly purified by density gradient centrifugation based on separation by size [5, 9, 16, 24, 28, 33, 50]. These methods result in particles with a purity greater than 80 % [19] but do not readily discriminate between VLPs and BVs [17, 47]. Therefore, BV is likely to be present in many of these preparations, and moreover, BVs have been shown to have an adjuvant effect—especially live BVs [27, 51]. Purification procedures based on chromatography have been shown to remove the contaminating BV in VLP preparations [35, 36, 43, 60, 61]. The purification technique affects the structural characteristics, immunogenicity, morphological integrity, antigenicity and functionality of purified VLPs [13, 28, 35, 45].

This study explores the effect of anion exchange chromatography purification on the homogeneity, morphology, antigenicity and immunological properties of NoV VLPs. In addition, several methods are applied for determining their purity.

Materials and methods

Production of NoV VLPs and mock baculoviruses

The insect cell line Sf9 (Invitrogen, Carlsbad, CA) was used to produce NoV GII-4 VLPs as described earlier [28]. A crude purification of VLPs was done using two discontinuous sucrose gradients, and sucrose was removed by overnight dialysis against phosphate-buffered saline (PBS) (pH 7.2). VLPs were concentrated using an Amicon Ultra 30 kDa centrifuge filter device (Millipore Corporation, Billerica, Germany).

Baculoviruses lacking the recombinant NoV gene (mock BV) were produced in Sf9 cells using a baculovirus expression kit (Invitrogen). The cells were seeded in Multidish 6-well plates (Nunc, Thermo Fisher, Scientific, Roskilde, Denmark) at a density of 1×10^6 cells/ml in serum-free medium (Sf 900 SFM III; Invitrogen) and transfected with bacmid DNA (1 μg) using Cellfectin (Invitrogen). The cells were grown at 27 °C and harvested 72 h post-transfection. The cell suspension was centrifuged at 500 × g for 5 min, and the supernatant (P1 BV stock) was collected and used to infect fresh Sf9 cells. Six days postinfection (dpi) the cell suspension was centrifuged as above, and the supernatant (P2 BV stock) was collected and stored at 4 °C. P2 BV stock was used to infect 200-ml cell cultures at a multiplicity of infection (MOI) of 1 and at 6 dpi, the culture was clarified by centrifugation at 3000 × g for 20 min at 4 °C. Mock BVs were concentrated by ultracentrifugation (L8-60M ultracentrifuge, Beckman SW-32.1 Ti rotor) at 100,000 × g for 1.5 h at 4 °C and resuspended in 0.2 M Tris–HCl, pH 7.3. BVs were loaded onto a 10 %–60 % discontinuous sucrose gradient and ultracentrifuged at 100,000 × g for 3 h at 4 °C. Fractions containing BVs determined by SDS-PAGE as described below were pooled, and sucrose was removed by overnight (o/n) dialysis against 1 liter of PBS. BVs were concentrated using an Amicon Ultra 50 kDa centrifuge filter device (Millipore Corporation) and stored at 4 °C in PBS.

Anion exchange chromatography purification of NoV VLPs

Chromatographic purification of NoV GII-4 VLPs was done using a column with anion exchangers (5 ml HiTrap Q, GE Healthcare, Uppsala, Sweden). The column was washed with start buffer consisting of 50 mM sodium phosphate (pH 6.6) to remove preservatives. Next, the column was washed with elution buffer consisting of 0.5 M NaCl in 50 mM sodium phosphate (pH 6.6) and equilibrated with start buffer. Crude purified NoV GII-4 VLPs in PBS were dialyzed against the start buffer before loading into the column (500 μg/column). Unbound VLPs were washed out with start buffer, and column-bound proteins were eluted using elution buffer. A total of 26 fractions (500 μl) were collected and analyzed by SDS-PAGE. The fractions containing NoV capsid were pooled and dialyzed against start buffer. The chromatographic purification was repeated using a fresh column. The fractions were collected, and the identity of the protein was confirmed by SDS-PAGE and immunoblotting. The fractions that were free of BV gp64 protein were pooled, dialyzed against PBS, and sterile filtered using a 0.2-μm syringe filter (VWR, Darmstadt, Germany).

The mock BV preparation was applied to the anion exchange chromatography column using the same method as described above. The fractions (26 fractions, 500 μl each) were collected and analyzed by SDS-PAGE.

SDS-PAGE and immunoblotting

Analysis of NoV capsid and BV proteins by SDS-PAGE was done using polyacrylamide gels with 12 % acrylamide

in the separating gel and 5 % in the stacking gel (Bio-Rad Laboratories, Hercules, CA). Samples were boiled for 5 min in Laemmli sample buffer containing 2 % SDS, 5 % β-mercaptoethanol, 62.5 mM Tris-HCl (pH 6.8), 25 % glycerol and 0.01 % bromophenol blue (Bio-Rad). Gels were stained with PageBlue™ Protein Staining Solution (Fermentas, Vilnius, Lithuania). For higher sensitivity, protein detection gels were stained with PageSilver™ Silver Staining Solution (Fermentas) according to the manufacturer's instructions. To determine the protein purity of NoV VLPs, densitometric analysis of Page Blue-stained SDS-PAGE gels was performed using AlphaEase® FC software (Alpha Innotech, San Leandro, CA). Mock BV and Sf9 cell lysate were used as controls.

For immunoblotting analysis, the proteins were transferred onto a nitrocellulose membrane (Trans-Blot transfer membrane, Bio-Rad). The NoV capsid proteins were detected using NoV GII-4 monoclonal antibody (Kim Laboratories Inc., Illinois, USA) at a 1:4000 dilution in 1 % milk and 0.05 % Tween 20 in Tris-buffered saline (TBS). The BV protein gp64 was detected using 1:400 dilution of anti gp64 monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, USA) in 1 % milk, 0.05 % Tween 20 in TBS. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Sigma-Aldrich) was used as a secondary antibody at a dilution of 1:10,000 to detect NoV capsid proteins, and at a 1:1000 dilution to detect the BV gp64 protein. The membranes were developed colorimetrically using an Opti-4CN detection kit (Bio-Rad) according to the manufacturer's instructions.

Determination of protein and total DNA content

The total protein content of the NoV VLPs was determined by Pierce BCA Protein Assay (Thermo Science, Rockford, USA) according to the manufacturer's instructions, with bovine serum albumin (BSA) used as a standard. The optical density (OD) at 544 nm was determined using a Victor^2 1420 Multilabel Counter (Perkin Elmer, Waltman, MA, USA).

The total dsDNA concentration was determined using a Quant-it dsDNA Broad-Range Assay Kit (Invitrogen) according to the manufacturer's instructions, with λ DNA used as a standard. Fluorescence at excitation/emission 485/535 nm was determined using a Victor^2 1420 Multilabel Counter (Perkin Elmer).

Determination of endotoxin content and sterility test

Endotoxin levels in the purified VLP preparations were quantified by Limulus amebocyte lysate (LAL) assay (Lonza, Walkersville, MD, USA) according to manufacturer's instructions.

In general, more than one culture medium is recommended for sterility testing [64]. Fluid thioglycolate medium and soybean-casein digest medium (both from Sigma-Aldrich) were used for VLPs sterility testing according to manufacturer's instructions.

Baculovirus DNA genome analysis

The amount of baculoviral DNA quantity was determined using a BacPAK™ qPCR Titration Kit (Clontech, CA, USA) according to manufacturer's instructions. Briefly, DNA was extracted using the NucleoSpin® Virus Kit included in the BacPAK™ qPCR Titration Kit. SYBR Green detection technology was used to determine baculoviral DNA genome content using the BV AcMNPV vector as a standard (detection range from 1.4×10^0 to 1.4×10^8 copies/μl). Pure NoV VLPs, crudely purified NoV VLPs (positive control) and Sf9 insect cell lysate (negative control) were serially diluted 10^0–10^{-2}. Forty-cycle PCR was run in a 7900HT Fast Real-Time PCR machine (Applied Biosystems, California, USA) using the 96-plate format with the following conditions: primary denaturation at 95 °C for 30 s, denaturation at 95 °C for 3 s and annealing/extension at 60 °C for 30 s. Because of DNA extraction and dilution of samples, a starting copy number value for the samples was back-calculated by the corresponding dilution factors according to manufacturer′s instructions.

Baculovirus titer determination

Live BV titers were determined using a BacPAK™ Rapid Titer Kit (Clontech). Early log-phase Sf9 cells were seeded on a 96-well microtiter plate (Nunc™, Roskilde, Denmark) in Sf900 cell medium (6.5×10^4 cells/well) and infected with serially diluted test samples. After a 1-hour incubation, the samples were aspirated from the wells, and the cells were covered with methyl cellulose. After incubation for 43–47 h at 27 °C, the cells were fixed with 4 % paraformaldehyde (Sigma Aldrich), stained with monoclonal gp64 antibody and detected with HRP-conjugate provided in the kit. Using an inversion microscope (Nikon, Badhoevedorp, The Netherlands), the foci of infection (clusters of infected cells) were counted in duplicate wells. Plaque-forming units per ml (ifu/ml) were calculated by multiplying the average number of foci per well by the corresponding dilution factors.

Electron microscopy (EM)

The VLP preparations were negatively-stained with 3 % uranyl acetate (UA) (pH 4.6). Three μl of the VLP sample was applied to a carbon-coated grid for 30 s. The grid was

dried with filter paper and 3 µl of UA was applied to the grid for 30 s. Excess liquid was removed, and the grid was examined using an FEI Tecnai F12 electron microscope (Philips Electron Optics, The Netherlands) operating at 120 kV.

Immunization of mice

Female BALB/c mice, aged 7 weeks (4 mice/group), were immunized twice intradermally (ID) with pure NoV VLPs at weeks 0 and 3. The doses were 1 µg and 10 µg per immunization point. Blood samples were drawn at study weeks 0 (pre-bleed), 2, 3 and 4, mice were euthanized 2 weeks after the second immunization, and whole blood was collected. Negative control mice were left unvaccinated.

Enzyme-linked immunosorbent assay (ELISA)

Sera from immunized and control mice were tested for NoV GII-4 VLP-specific IgG, IgG1, and IgG2a antibodies by enzyme-linked immunosorbent assay (ELISA) as described in detail elsewhere [10, 58]. Briefly, GII-4 VLPs were coated at 0.2 µg/ml (100 µl/well). Serum samples were used at 1:200 dilution or twofold dilution series were utilized. Analyses of IgG1 and IgG2a antibodies were done using pooled sera from mice. Horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Sigma-Aldrich) was used at a dilution of 1:4000. IgG subtypes were determined using goat anti-mouse IgG1 or IgG2a HRP conjugates (Invitrogen) at a dilution of 1:6000.

Results

To identify the impurities in the NoV VLP preparation after crude purification with two successive sucrose gradients [28], various analyses were performed. First, NoV capsid proteins were analyzed by 12 % SDS-PAGE and Page Blue staining (Fig. 1A). As seen from the gel, NoV capsid protein appeared as a doublet protein band (58 and 64 kDa), which is typical for the capsid GII-4 protein [28]. In addition, a weaker band in the preparation of NoV capsid VLPs and mock BV, indicated by an arrowhead, was detected, corresponding to the size of the BV envelope gp64 protein (Fig. 1A, lanes a and b). No other proteins derived from BV or Sf9 cells were observed in the VLP preparation. Confirmation of protein identity in the VLP preparation was performed by immunoblotting using NoV GII-4-specific and BV gp64-specific monoclonal antibodies (Fig. 1B). The specificity of the staining was confirmed using a mixture of these monoclonal antibodies to stain a Sf9 cell lysate, with a negative result. In addition, EM analysis of the protein samples (Fig. 1C), in addition to NoV VLPs of approximately 40 nm in size, revealed the presence of residual rod-shaped BVs in the preparation.

To determine the elution times for NoV VLPs and mock BV, both samples were applied to the anion exchange chromatography columns under identical conditions. Examination of the fractions collected (500 µl each) showed that an apparent peak (fractions 15–17, respectively) of the NoV capsid protein (Fig. 2A) was eluted just prior to the BV protein (Fig. 2B). Fractions 15–17 were completely negative when analyzed by immunoblot using BV gp64-specific monoclonal antibody, indicating the absence of BV contamination (data not shown). Further optimization of the steps of the purification process were undertaken to improve the yield of the pure NoV capsid protein. First, different concentrations of NaCl (0.5 M, 1 M and 1.5 M) were used to test the effect of the ionic strength of the elution buffer (50 mM Na_2HPO_4). Second, the concentration of the protein sample loaded onto the column was also optimized. The best purity and yield were obtained with 50 mM Na_2HPO_4-0.5 M NaCl buffer, and the optimum amount of crude purified VLPs was 500 µg/column (data not shown). After the first anion exchange chromatography step, fractions 15–17 were pooled and applied to the second, fresh column. The analysis of collected fractions of the second anion exchange chromatography is shown in Fig. 3A. The three fractions (15–17) containing NoV VLPs were collected, pooled, dialyzed against PBS and sterile-filtered. Immunoblotting of each fraction confirmed the lack of BV gp64 impurities (Fig. 3B).

From the starting material (2 mg crude purified VLPs), 0.226 mg anion-exchange-chromatography-purified NoV capsid VLPs was obtained and subjected to several analyses (Figs. 4, 5; Table 1). The identity of the NoV VLPs was demonstrated by immunoblotting using an NoV GII-4-specific monoclonal antibody (Fig. 4A). The results showed doublets of NoV capsid proteins [28] without detectable degradation products. Probing with a BV-gp64-specific monoclonal antibody did not detect any BV gp64 protein (Fig. 4B). Densitometric analysis of the SDS-PAGE confirmed that the NoV VLPs were >90 % pure. The presence of live BV was analyzed as well. As seen in Fig. 4C, duplicate wells of the purified VLP sample completely lacked live BVs in contrast to the mock BV sample wells, which contained $\sim 10^9$ pfu/ml live BV, as determined using a BacPak Rapid Titer Kit. The total DNA content in the pure VLP sample was also quantified, and some residual DNA was detected, but the DNA content was very low (0.41 ng/µl). In addition, the genomic BV DNA was quantified using BV Q-PCR. BacPAK control BV DNA template was used to create a standard curve (Fig. 5). Purified NoV VLPs contained only a few copies of

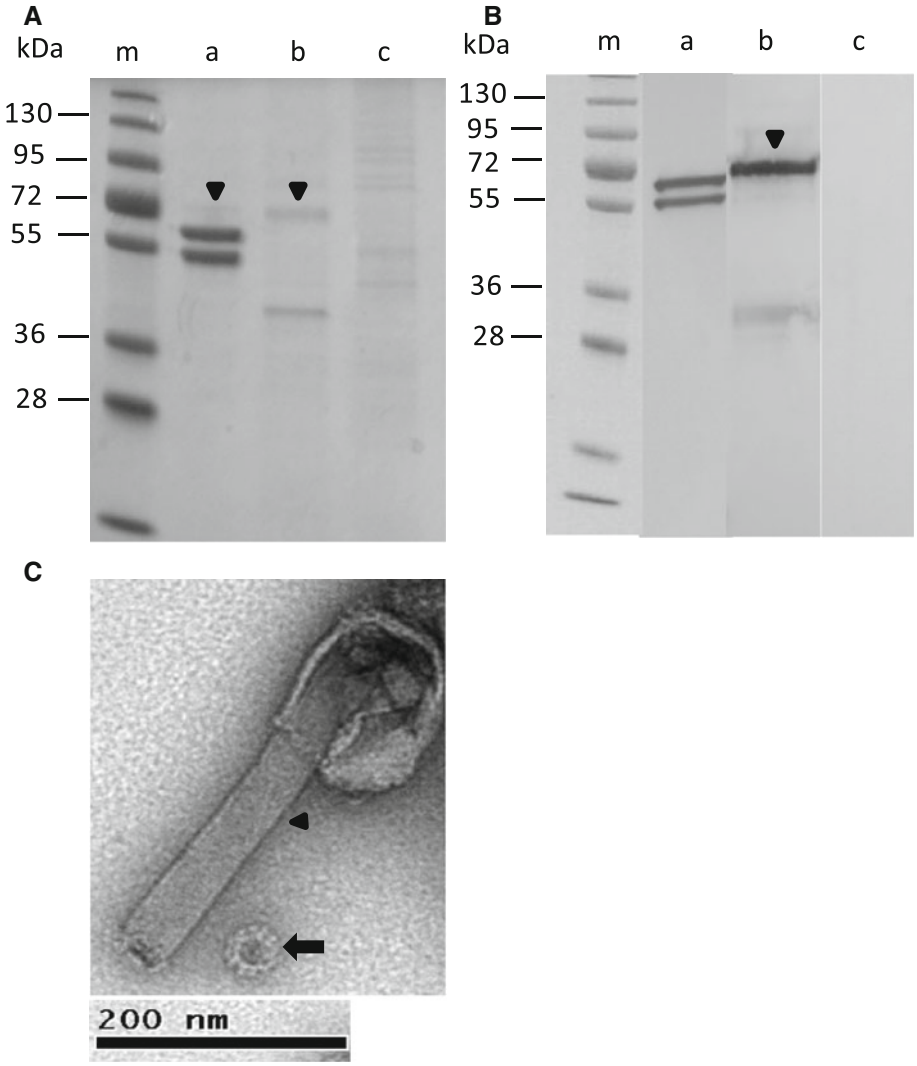

Fig. 1 Analysis of the identity and purity of sucrose-gradient-purified NoV capsid VLPs. (**A**) Protein analysis by 12 % SDS-PAGE. Lane m, protein molecular weight marker; lane a, NoV capsid protein VLPs; lane b, mock BV; lane c, Sf9 insect cell lysate. (**B**) Immunoblotting of NoV capsid protein shown in panel A with NoV GII-4- (lane a) and BV gp64-specific (lane b) monoclonal antibodies. An Sf9 insect cell lysate (lane c) served as a negative control and was probed with both NoV- and BV-specific monoclonal antibodies. An arrowhead in panels A and B indicates the major band of the BV gp64 protein. (**C**) Electron microscopic analysis of a gradient-purified VLP preparation at a magnification of ×18,500. The arrow indicates an NoV VLP, and the arrowhead, residual BV

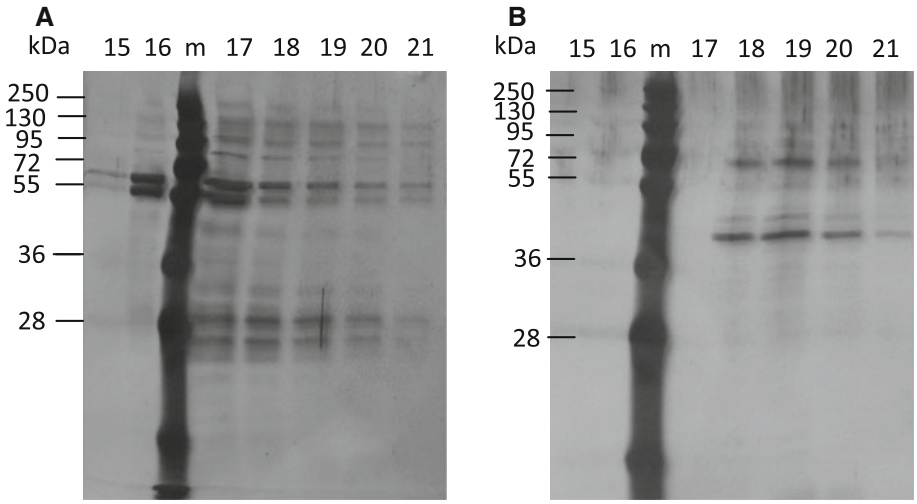

Fig. 2 Anion exchange chromatography fractions 15-21 analyzed by 12 % SDS-PAGE and visualized by silver staining. (**A**) Fractions from NoV capsid VLPs and **(B)** fractions from control mock BV run through the chromatography columns. m, protein molecular weight marker

the BV DNA (Table 1; Fig. 5). From the copies of BV DNA in a pure sample, the amount of BV DNA was calculated to be <0.001 pg/µl.

EM examination of the purified samples revealed that the structural characteristics (protein assembly into VLPs) and morphological integrity were not affected by the purification

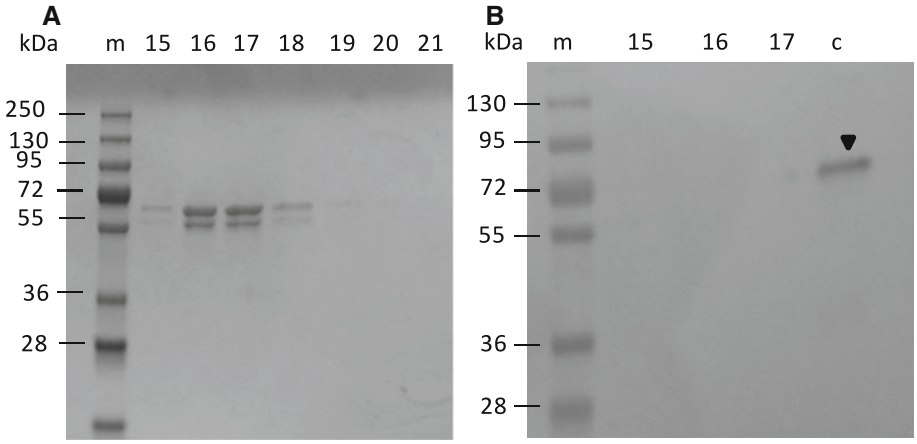

Fig. 3 Anion exchange chromatography fractions 15–21 of NoV capsid VLPs after the second column purification analyzed by SDS-PAGE and visualized by Page Blue (**A**) Immunoblotting of fractions 15-17 using BV-gp64-specific monoclonal antibodies (**B**) Mock BV was used as positive control (lane c). An arrowhead indicates BV. m, protein molecular weight marker

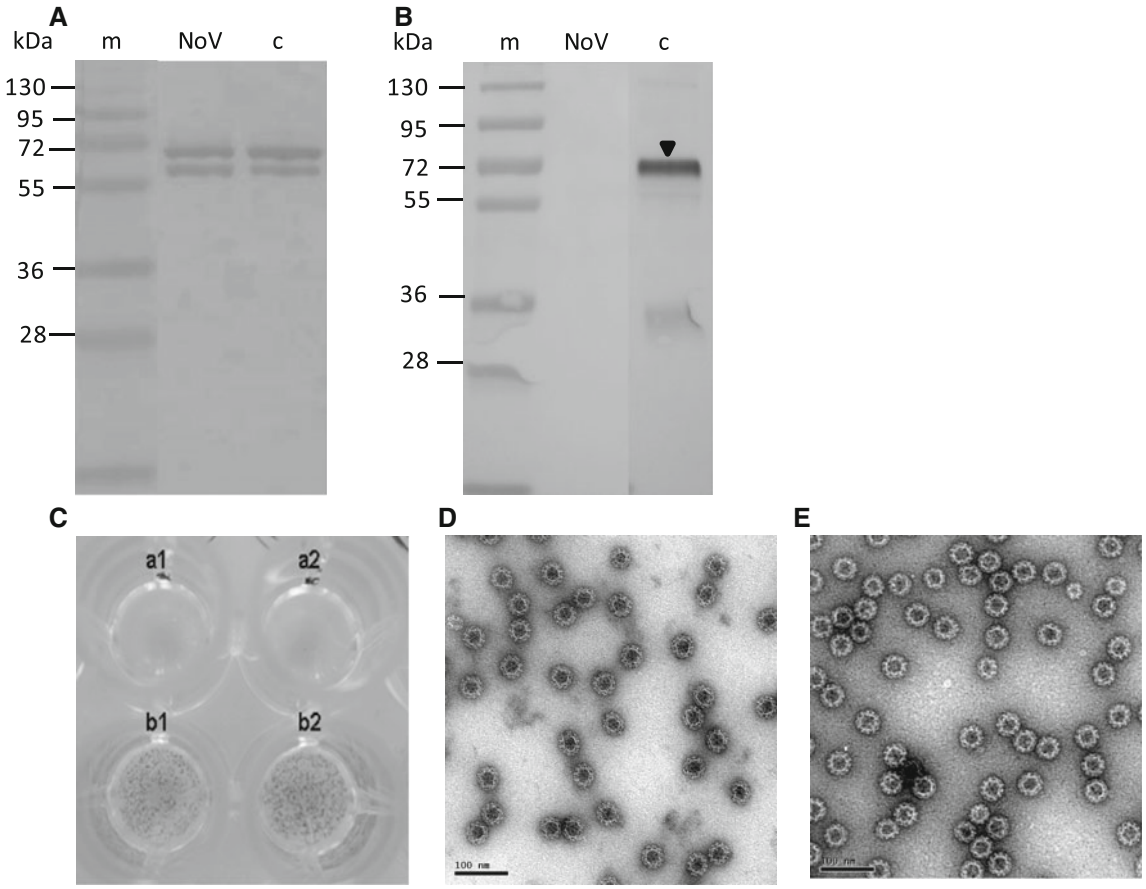

Fig. 4 Identity, purity and morphology of anion-exchange-chromatography-purified NoV capsid VLPs. Immunoblot analysis of (**A**) pure NoV VLPs detected by GII-4-capsid-specific and (**B**) BV-gp64-specific monoclonal antibodies. Sucrose-gradient-purified NoV VLPs were used as a control (lane c). An arrowhead indicates BV. m, protein molecular weight marker. (**C**) Live BV detection using a BacPAK^{TM} Rapid Titer Kit. Replicate wells a1 and a2 contain an anion-exchange-chromatography-purified NoV VLPs sample. Dark spots (foci) in the b1 and b2 wells indicate a sample positive for live BV (mock BV). EM of chromatographically purified NoV VLPs (**D**) and sucrose gradient purified NoV VLPs (**E**) observed at a magnification of ×30000. Bar, 100 nm

process (Fig. 4D) and were similar to those of the gradient-purified VLPs (Fig. 4E). Chromatographically purified NoV VLPs of approx. 40 nm in size were seen in every grid square. NoV VLPs were not contaminated with bacterial endotoxin, as we detected <0.1 EU/10 μg of protein; an amount that is below the international standard of ≤30 EU/20 μg of protein [41]. The purified VLPs were sterile and stable for at least 5 months at 4 °C in PBS, pH 7.4 (data not shown).

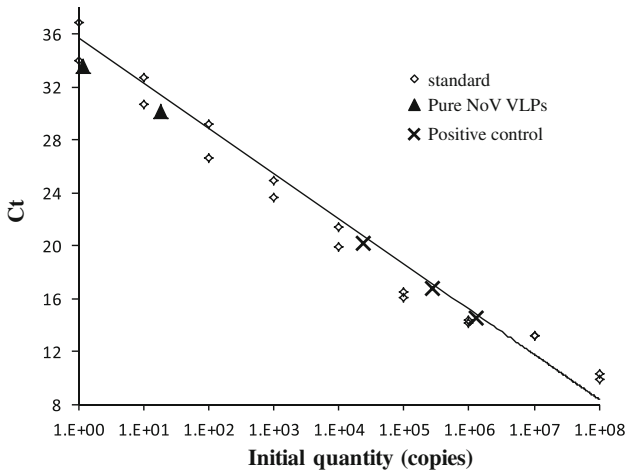

Fig. 5 Baculovirus Q-PCR. Standard curve created using serial dilutions of the BacPAK control template (1.4×10^8–1.4×10^0), demonstrating a strong correlation between Ct values and the DNA copy number (log scale) with R2 = 0.975, slope = −3.20, y-Intercept = 34.15. Pure NoV VLP samples, positive control samples (crude purified NoV VLPs) and negative control sample (Sf9 insect cell lysate) were serially diluted 10^0–10^{-2}. The number of copies BV DNA in the negative control sample were under the detection limit of the assay

Table 1 Specification and analysis of expression system impurities in the NoV GII-4 capsid VLPs purified by anion chromatography

Test	Specification	Detection assay
Protein concentration	0.452 mg/ml	Pierce BCA Protein Assay
Yield*	0.226 mg	
Identity	58–64 kD protein detected**	SDS-PAGE and immunoblot
Purity	>90 %	Densitometric analysis
Plaque-forming units (pfu)***	0 pfu/ml	BacPAK Rapid Titer Kit
Morphology	VLPs (~40 nm)	Electron microscopy (EM)
DNA content	0.41 ng/μl	Quant-it dsDNA Broad-Range Assay Kit
BV DNA	3.31 copies/μl (<0.001 pg/μl)	BacPAK qPCR titration kit
Endotoxin	<0.1 EU/10 μg of protein	Limulus amebosyte lysate assay
Bioburden	No growth observed	Sterility test

* Total yield after chromatographic purification of 2 mg staring material

** [28]

*** Live BV titer

To determine the effect of anion exchange chromatographic purification on the immunogenicity of the VLPs, BALB/c mice were immunized with pure NoV GII-4 VLPs at 1 and 10 μg doses without external adjuvants. NoV-GII-4-specific serum antibody responses were induced by each dose of pure NoV VLP (Fig. 6). The IgG response was already quite high after the first immunization and was increased after the second dose at three weeks (Fig. 6A). NoV-specific IgG subtypes were measured to determine the Th1 and Th2 responses [10]. All test groups had similar levels of NoV-specific IgG1 (Fig. 6B) and IgG2a (Fig. 6C) antibodies. Negative control mice lacked detectable NoV-specific antibodies.

Discussion

NoV VLPs are commonly used in different serological assays and animal immunogenicity studies [7, 24, 25, 30]. Density gradient purification of VLPs based on size discrimination is most commonly used in laboratory research [28, 52]. These gradient-purified VLPs contain recombinant BVs and other impurities originating from the expression system [19]. BVs are multiplied to very high titers in suspension culture, complicating the purification procedure. We used two successive anion exchange chromatography steps to purify the VLPs. Our data demonstrate that chromatographically purified VLPs derived from NoV genotype GII-4 possess excellent purity, morphology, antigenicity and immunogenicity.

An adjuvant effect on the immune system of live BVs present in the VLPs has been suggested, specifically in parvovirus and influenza virus VLP preparations [26, 27, 52]. We investigated BV-derived impurities in the sucrose-gradient-purified NoV GII-4 VLPs. The major protein impurity (close to 10 %, determined by densitometric analysis) came from envelope glycoprotein gp64 of BV. The BV gp64 is also linked to adjuvant behavior [2, 23]. gp64 contains a mannose-binding residue, which is expressed on macrophages and dendritic cells. Abe et al. [2] have suggested that gp64 recognizes a Toll-like receptor (TLR) and activates the immune response by inducing inflammatory cytokines such as tumor necrosis factor-γ (TNF-γ) and interleukin-6 (IL-6). The anion-exchange-chromatography-purified NoV VLP preparations were examined for expression-vector-related protein impurities. Densitometrical SDS-PAGE analyses as well as immunoblotting identified NoV capsid protein with >90 % purity and without the traces of BV gp64 protein.

To obtain extremely pure VLPs, several chromatography steps are required [35, 45, 63], but repeated chromatography steps can affect the conformation of VLPs [11]. Purification procedures that preserve the intact conformation of VLPs are important because recombinant VLPs are inherently unstable and tend to denature and aggregate in solution [11, 55]. We used a purification procedure with a combination of two anion exchange chromatography steps in order to minimize

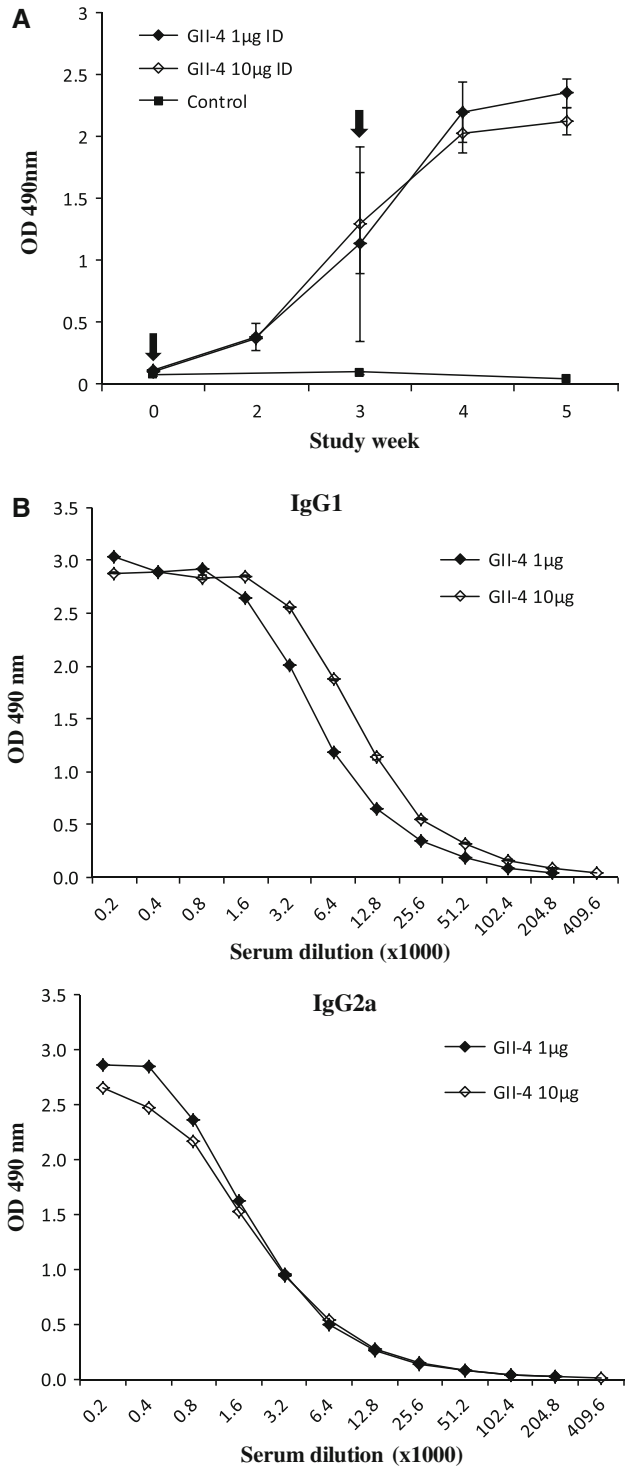

Fig. 6 NoV capsid-specific antibody responses of BALB/c mice immunized twice intradermally (ID) with 1 µg or 10 µg of pure NoV GII-4 VLPs. (**A**) Serum samples collected at study weeks 0 (pre-bleed), 2, 3, 4 and 5 were tested by ELISA for NoV-specific IgG. The arrows indicate immunization points at study weeks 0 and 3. NoV-specific serum IgG1 (**B**) and IgG2a (**C**) subtype antibody responses. Mean ODs of the groups with standard errors are shown. Control mice were left unvaccinated

impurities and to obtain structurally intact NoV VLPs. Our results show that NoV VLPs purified by two anion exchange chromatography steps retain the structure of the icosahedral capsids and show excellent stability, comparable to density-gradient-purified VLPs (Fig. 4D, E).

It has been reported that ion exchange chromatography is the process of choice for removing to the flowthrough pool host-cell protein and DNA [34, 60, 65]. The anion exchange chromatography method is based on adsorption and reversible binding of charged samples. The major DNA impurities in the VLP preparations come from the baculoviral DNA and not the host cells [27]. Abe and colleagues [1] have shown that BV DNA contains unmethylated CpG motifs that induce proinflammatory cytokines through the TLR-9/MYD88-dependent signaling pathway. Another group has also reported that BV DNA may be responsible for the adjuvant properties of BVs [27]. Our results show that the number of copies of BV DNA was extremely low (Fig. 5; Table 1) in the pure NoV VLP preparation. To the best of our knowledge, this is the first study to determine the number of copies of BV DNA in an NoV VLP preparation by the quantitative BV Q-PCR method. The total dsDNA content in the pure NoV VLP preparation was low (0.9 ng/1-µg dose). In the case of VLP-based vaccine production, the residual DNA needs to be removed to reach acceptable threshold values, typically 10 ng/dose [15]. One group has shown that only live BV was able to stimulate innate immunity, which was not due to the presence of viral DNA [23]. Therefore the presence and quantity of live BVs in the pure VLP preparation were determined. The infectious BV titer was 0 pfu/ml, confirming the complete absence of recombinant live BV in the preparations of pure NoV VLP.

BALB/c mice were immunized with pure NoV VLPs with two relatively low doses. A strong NoV-GII-4-specific immune response was induced. Balanced Th1-type and Th2-type immune response (reflected as IgG2a and IgG1 antibody production) [10] was generated with the VLPs in the absence of external adjuvants. Although we performed no head-to-head comparison of immunogenicity of classical density-gradient-purified and chromatographically purified NoV VLPs, the results obtained in the present study are comparable to those obtained previously with a crude preparation of purified VLPs [10, 58]. These results indicate that pure NoV VLPs are extremely potent immunogens. Others have also shown that compared to soluble individual proteins, multivalent structures of VLPs induce strong B cell and T cell responses in the absence of adjuvants [19, 31].

Finally, we have described a relatively straightforward chromatography method for purification of NoV VLP and

methods to confirm their purity. The purified VLPs were tested for impurities including BV protein, DNA, live BV and other impurities related to the expression system. Most significantly, the purified NoV capsid VLPs retained their morphological and antigenic features as well as their immunogenicity. Recombinant NoV VLP vaccine candidates are in the early phases of development [4, 10, 59]. Some research groups have used intranasally delivered NoV VLPs in small animals as well as in human challenge studies [4, 59]. In addition, a vaccine against acute gastroenteritis in young children containing a combination of NoV VLPs and rotavirus VP6 protein is under development [10]. We suggest that this small laboratory-scale purification procedure may potentially be applied for large-scale production of NoV VLPs.

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Conflict of interest None of the authors have any conflict of interest.

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