Norovirus Epidemiology

Prevalence, transmission,
and determinants of disease susceptibility

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Cover: Norovirus particles as viewed in an electron microscope, with part of the cDNA code for norovirus in the background.

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To Sarah
Abstract

Norovirus (NoV) is today recognized as the most important agent of acute human gastroenteritis, causing a high number of diarrheal episodes in both adults and children. Outbreaks in hospitals, nursing homes, day-care centers, and from consumption of contaminated food and drinking water are common. Wastewater can be a source of NoV dissemination, e.g. when used for irrigation of crops, or due to shellfish cultivation near the outlet of wastewater treatment plants. Today, at least 25 different genotypes of NoV belonging to two major genogroups (GG) have been observed in humans. These genotypes are associated with different transmission patterns and disease severity in humans. Also host genetic factors, such as presence of ABO antigens and mutations in the FUT2 gene affect susceptibility, and can even render complete resistance to symptomatic infections, but only the most common NoV genotypes have been studied regarding this. In this thesis, we wanted to find prevention strategies for NoV disease through four studies of NoV epidemiology: Development of a sensitive real-time PCR assay for detection and quantification of human NoVs, characterization of NoV in children with diarrhea in Nicaragua, investigation of the prevalence and parameters influencing NoV concentration in a wastewater treatment plant in Gothenburg, Sweden, and studying host susceptibility factors in a foodborne NoV outbreak in Jönköping, Sweden.

First we developed a real-time PCR assay which can detect and quantify NoV in various settings, both in stool samples of patients, and in wastewater samples from which virus was first concentrated using ultracentrifugation. This assay was found to be more sensitive than commercial immunological assays and conventional PCR methods. The assay is furthermore able to differentiate between the two major human genogroups of NoV using melting curve analysis, which provides valuable information about the circulating NoV strains.

The survey of NoV in pediatric diarrhea in Nicaragua revealed a large impact of NoV, both in community and hospital based settings, with 15% of the severe diarrhea cases attributed to NoV. Peaks of clinically diagnosed NoV gastroenteritis were associated with emerging variants of genotype GGI.I.4, largely replacing the many different NoV genotypes circulating before the peak of diarrheal cases. Children infected with the GGI.I.4 genotype were found to shed more virus compared to children infected with other genotypes, which could partly explain the high transmission of GGI.I.4.
At the wastewater treatment plant in Gothenburg, both NoV GGI and GGII were detected during a whole year, not only during the winter season when clinical cases are common. This indicates that NoV infections are frequently occurring at clinical and/or sub-clinical levels in the community. During winter, GGII was present in high concentrations, whereas GGI concentration increased to higher levels than GGII in summer, possibly due to the emergence of new genotypes following the winter outbreaks. The levels of NoV GGI were stable during the year, and hence incoming concentrations were affected by dilution factors such as rain. Primary treatment and treatment in a conventional, non-nitrifying activated sludge system reduced the NoV concentration by a factor of about 30. The detection of NoV in outgoing water, together with the low reduction and lack of correlation to indicator bacteria, suggest that better monitoring tools for virus in wastewater are warranted to reduce environmental contamination.

A foodborne NoV outbreak in Jönköping in October 2007, by a NoV GGI.3 strain, revealed a surprising pattern of host susceptibility. In contrast to previous findings, this strain infected individuals irrespective of secretor status and Lewis (Le) phenotype, with non-secretors and Le\(^{a+b}\) individuals having a higher risk of disease. Individuals with blood group B had a partial protection to symptomatic infection, but none of the host factors investigated mediated complete resistance. Furthermore, we observed differences in susceptibility regarding homozygosity and heterozygosity in the FUT2 gene, with heterozygous secretor-positive individuals being more susceptible to symptomatic NoV infection than homozygous secretors.

In summary, the developed LUX real-time PCR assay was successfully used in all studies in this thesis, which yielded important information about the prevalence and transmission of NoV. We observed the emergence of GGII.4 variants, causing the majority of diarrheal cases in children, largely replacing the other circulating genotypes, possibly due to better replication leading to a higher viral shedding. After the peak of NoV-induced diarrheal episodes, the incidence of GGII.4 decrease and other strains emerge which can infect people not previously exposed. This was observed in the foodborne outbreak in Jönköping, where individuals expected to be resistant to NoV were infected, and indeed had a higher risk of developing disease. A similar seasonal pattern was also indirectly observed in wastewater, with high levels of GGII in winter, which subsequently declined, followed by an increase of GGI in summer. Taken together, these results provide a better insight into the epidemiology of the virus, which hopefully can lead to better preventive measures for NoV gastroenteritis.
Populärvetenskaplig sammanfattning

Norovirus (NoV) orsakar den s.k. vinterkräksjukan vilken är den vanligaste formen av akut mag-tarm inflammation i världen. Symptomen kan variera men består oftast av våldsamma kräkningar och diarré. Årligen infekteras över 250 miljoner människor med NoV vilket leder till att ungefär 200 000 barn dör, de flesta i utvecklingsländer. NoV orsakar ofta stora sjukdomsutbrott i slutna miljöer som sjukhus, vårdhem, dagis, kryssningsfartyg o.s.v. där viruset lätt kan spridas från person till person. Viruset är mycket motståndskraftigt mot desinfektionsmedel och temperaturskillnader och därför kan man lätt få vinterkräksjukan genom kontaminerad mat och dryck. Avloppsvatten kan vara en källa till infektion, exempelvis då skaldjursodlingar eller badvatten befinner sig i närheten av avloppsvattenutsläpp och därigenom kontamineras av viruset. NoV är ett mycket genetiskt instabilt virus med ett flertal underarter som har visat sig orsaka olika svåra symptomer. Ungefärlig en femtedel av Europas befolkning är vanligen resistent mot vinterkräksjukan men denna resistens är bara kartlagd för några få av alla underarter av NoV. Den höga smittsamheten, stabiliteten och det stora antalet underarter försvårar möjligheterna att begränsa eller hindra NoV infektioner. I min avhandling har jag därför studerat NoV utifrån olika perspektiv för att få klarhet i virusets förekomst och spridning i samhället:

- Utveckla en metod för att kunna påvisa och mäta mängden NoV i patientprover och i avloppsvatten
- Bestämma förekomst av NoV hos barn med diarré i Nicaragua samt hur olika faktorer påverkar svårighetsgrad av symptom
- Undersöka årlig förekomst och faktorer som påverkar mängden NoV i avloppsvatten vid reningsverket Ryaverket i Göteborg
- Kartlägga genetiska faktorer hos människan som påverkar mottaglighet för NoV infektion vid ett utbrott orsakat av kontaminerad mat i Jönköping

Vi utvecklade först en känslig molekylärbiologisk metod (realtids-PCR) som kan påvisa och mäta NoV koncentration i patientprover samt i avloppsvatten. Utöver detta kan metoden särskilja de två stora huvudgrupperna av NoV, vilket ger värdefull information om vilka underarter av NoV som cirkulerar och orsakar sjukdom. Denna metod användes sedan i de tre följande projekten.
I Nicaragua observerade vi att NoV orsakade en stor del av diarréfallen hos barn. Hela 15% av de allvarliga diarréer som resulterade i anmälan på sjukhus var orsakad av NoV. De stora ökningarna av diarré under året berodde på en utveckling av nya varianter av en specifik underart av NoV (GGII.4) som verkar mer smittsam samt ger svårare symptom. Barn som var infekterade med denna underart utsöndrade även mest virus, vilket delvis kan förklara dess höga smittsamhet.

I avloppsvattnet från Ryaverket i Göteborg påvisades NoV under ett helt år, inte bara under vinterhalvåret då kliniska fall är vanliga. Under sommarmånaderna dominerade mindre vanliga underarter i avloppsvattnet, medan de vanligare underarterna främst observerades under vintern. Mängden virus reducerades i genomsnitt 30 gånger under reningsprocessen i Ryaverket, vilket var likvärdigt med den genomsnittliga reduktionen av bakterier. Inget klart samband fanns dock om man jämförde bakterie och virus reduktion mellan enskilda månader.

Vid en konferens i Jönköping i oktober 2007 insjuknade många av deltagarna i kräkningar och diarré. Vi upptäckte att det berodde på att maten var kontaminerad med NoV av en ovanlig underart som sällan observeras. Övriga personer i regel är resista mot vinterkräksjukan insjuknade i stor utsträckning. Ingen av de undersökta genetiska faktorerna gav ett totalt skydd mot infektion, men personer med blodgrupp B insjuknade i mindre utsträckning jämfört med personer som hade blodgrupp A eller 0.

Sammanfattningsvis så utvecklade vi en metod för att mäta koncentration av NoV som sedan framgångsrikt användes i alla våra studier. Resultaten tyder på att NoV orsakar många allvarliga diarréer hos barn och att det mestadels är varianter av en specifik underart, GGII.4, som orsakar sjukdom under vinterkräksjukeperioden, dvs. vinterhalvåret i Europa och regnsäsongen i tropiska länder. Under andra halvan av året minskar förekomsten av GGII4 och andra underarter av NoV börjar cirkulera. Ett liknande mönster såg vi i avloppsvattnet där de mindre vanliga underarterna fanns i höga halter under sommaren. Dessutom fann vi att vissa underarter av NoV kan infektera personer som ofta är resista mot vinterkräksjukan. Våra resultat ger en större förståelse av virusets förekomst och spridningsvägar och kan förhoppningsvis leda till bättre förebyggande åtgärder mot vinterkräksjukan i framtiden.
List of papers
The papers included in this thesis are listed below. They will be referred to in the text by their roman numerals.

I  Nordgren, J., Bucardo, F., Dienus, O., Svensson, L., and Lindgren, P-E. Novel light-upon-extension real-time PCR assays for detection and quantification of genogroup I and II noroviruses in clinical specimens


III  Nordgren, J., Matussek, A., Mattsson, A., Svensson, L., and Lindgren, P-E. Prevalence of norovirus and factors influencing virus concentrations in a full scale wastewater treatment plant

IV  Nordgren, J., Kindberg, E., Lindgren, P-E., Matussek, A., and Svensson L. A FUT2 nonsense mutation (G428A) and Lewis-independent norovirus GI.3 outbreak
Submitted manuscript.

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# Table of contents

ABSTRACT .............................................................................................................................................................. V  
POPULÄRVETENSKAPLIG SAMMANFATTNING................................................................................................... VII  
LIST OF PAPERS .................................................................................................................................................. IX  
ABBREVIATIONS ................................................................................................................................................ XII  

INTRODUCTION .................................................................................................................................................. 1  
1. VIRAL GASTROENTERITIS .............................................................................................................................. 3  
   1.1 GENERAL INTRODUCTION TO DIARRHEAL DISEASE ............................................................................. 3  
   1.2 THE VIRAL PATHOGENS ......................................................................................................................... 4  
   1.3 THE EMERGING IMPORTANCE OF NOROVIRUS .................................................................................. 5  

2. DIVERSITY AND CLASSIFICATION OF NOROVIRUS .................................................................................. 7  
   2.1 A FAMILY WITH MANY MEMBERS ..................................................................................................... 7  
   2.2 STRUCTURE .............................................................................................................................................. 8  
   2.3 DETECTION AND MOLECULAR CHARACTERIZATION ........................................................................... 9  

3. TRANSMISSION OF NOROVIRUS IN THE HUMAN ENVIRONMENT ........................................................... 11  
   3.1 DIFFERENT PROPERTIES OF NOROVIRUS STRAINS .......................................................................... 11  
   3.2 CLINICAL SYMPTOMS AND FACTORS FACILITATING THE SPREAD OF DISEASE ................................ 13  
   3.3 NOROVIRUS IN WASTEWATER ................................................................................................................ 14  

4. WHY ARE SOME PEOPLE RESISTANT TO WINTER VOMITING DISEASE? .................................................. 16  
   4.1 A BRIEF HISTORY OF THE GENETIC FACTORS DETERMINING SUSCEPTIBILITY ............................ 16  
   4.2 HISTO-BLOOD GROUP ANTIGENS AS THE PROBABLE RECEPTORS FOR NOROVIRUS ........................ 16  

5. AIMS .............................................................................................................................................................. 19  

6. DEVELOPMENT OF A NEW METHOD FOR DETECTING HUMAN NOROVIRUSES (PAPER I) ...................... 21  

7. CHARACTERIZATION OF NOROVIRUS IN CHILDREN’s DIARRHEA (PAPER II) ........................................ 24  

8. HIGH PREVALENCE OF NOROVIRUS IN A WASTEWATER TREATMENT PLANT (PAPER III) .................. 27  

9. NEW SUSCEPTIBILITY PATTERNS REVEALED IN A FOODBORNE NOROVIRUS OUTBREAK (PAPER IV) ... 30  

10. CONCLUDING REMARKS .......................................................................................................................... 33  

ACKNOWLEDGEMENTS .................................................................................................................................. 35  
REFERENCES .................................................................................................................................................. 37  
APPENDIX A: PCR PRIMERS ................................................................................................................................ 45
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>AGE</td>
<td>Acute gastroenteritis</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EM</td>
<td>Electron microscopy</td>
</tr>
<tr>
<td>FUT2</td>
<td>Fucosyltransferase 2</td>
</tr>
<tr>
<td>GGI, GGII</td>
<td>Genogroup I and II respectively</td>
</tr>
<tr>
<td>HBGA</td>
<td>Histo-blood group antigen</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo Dalton</td>
</tr>
<tr>
<td>Le a, b</td>
<td>Lewis antigen a, b</td>
</tr>
<tr>
<td>NoV</td>
<td>Norovirus</td>
</tr>
<tr>
<td>NSP</td>
<td>Non-structural protein</td>
</tr>
<tr>
<td>OR</td>
<td>Odds ratio</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>RdRp</td>
<td>RNA-dependent RNA polymerase</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse-transcription</td>
</tr>
<tr>
<td>VLP</td>
<td>Virus-like particle</td>
</tr>
<tr>
<td>VP</td>
<td>Viral protein</td>
</tr>
<tr>
<td>WWTP</td>
<td>Wastewater treatment plant</td>
</tr>
</tbody>
</table>
Introduction

Norovirus (NoV), causing the winter vomiting disease, is today recognized as the most important agent of acute human gastroenteritis, causing ~270 million infections and an estimate of 200,000 deaths in children annually. Second only to rotavirus in endemic pediatric diarrhea, NoV is by far the most frequent pathogen in epidemic diarrhea in both developing and industrialized countries, with outbreaks frequently occurring in closed settings such as hospitals and day-care centers. The high stability and infectivity of the virus moreover make it a common cause of food and waterborne gastroenteritis. Despite these facts, there are no vaccines or therapeutic treatments available for this disease. Moreover, little is known about the transmission pathways of the virus, and which measures that could be taken in order to prevent contamination of the environment. This is partly due to the complex NoV epidemiology, with several strains circulating, each having their own intrinsic properties which are related to differences in severity, transmission pathways, and receptor specificities in the human host.

Thus, before being able to find treatments or prevention strategies for this disease, many questions about the epidemiology need to be answered. Are there certain strains of NoV that are more clinically important, i.e. causing the majority of the severe gastroenteritis? What are the mechanisms behind the seasonality, with most clinical cases appearing in the winter months? How does the virus maintain its circulation in humans after the winter season? How can we reduce the environmental contamination, e.g in water treatment plants to prevent outbreaks? Which are the human receptors, and how do they differ between the various NoV strains? This thesis aims at giving answers to some of these questions.
1. Viral gastroenteritis

1.1 General introduction to diarrheal disease
Despite improved water and food handling (1), diarrhea is still a common illness worldwide, accounting for ~2 million deaths in children <5 years of age (2), mainly in developing countries. Diarrhea is thus the largest killer after pneumonia and neonatal deaths (2), causing approximately 17% of the fatalities (Figure 1).

Diarrheal diseases also cause significant morbidity and economic loss (3); in children <5 years, estimates’ indicate that more than 700 million cases of acute diarrhea occur each year (4). There are many bacterial and parasital pathogens causing diarrhea, some of the most common include enterotoxigenic and enteropathogenic E.coli, Shigella spp, Campylobacter jejuni, Salmonella spp, Entamoeba histolytica and Giardia (5-7). Historically, the bacterial pathogens have received most attention, both due to lack of knowledge of, and sensitive detection methods for, viral pathogens (8-10). Indeed, it was not until 1972 that the first virus causing gastroenteritis was isolated (see section 1.2). However, as better tools developed for the detection of viruses (8, 11-13), this view has changed dramatically. It is now widely accepted that viruses are the cause of the majority of both sporadic and epidemic gastroenteritis episodes world-wide (14-17). Also, many cases of gastroenteritis remain of unknown etiology (9, 17), and it is likely that the majority of them are caused by viruses. The rates of bacterial food borne illnesses in the industrialized world have declined, partly as a result of improved refrigeration (1), a measure ineffective against many viruses (18), which
will likely increase the percentage of virus-induced gastroenteritis in the future. Bacteria are estimated responsible for only <5% of the diarrheal cases in industrialized countries, whereas in developing countries, bacteria are responsible for approximately 25% of the cases occurring in children <2 years of age requiring hospitalization (19).

1.2 The viral pathogens
There are four major viral groups causing diarrhea: rotavirus, calicivirus (NoV and sapovirus), astrovirus and adenovirus (serotypes 40, 41) (Table 1). Other, less common viruses include toroviruses, picobirnaviruses, picornavirus (the Aichi virus), coronavirus, pestivirus and enterovirus 22 (10, 20, 21). These four major families all cause endemic childhood disease; this illness affects all children worldwide within the first few years of life regardless of their level of hygiene, quality of water, food or sanitation (20). Rotavirus is the most common endemic childhood pathogen, resulting in 2 million hospitalizations each year (15), ~39% of all childhood diarrhea hospitalizations (22), and approximately 611,000 deaths (22). Thus, rotavirus has received enormous attention during the last decades, which has lead to the development and licensing of two new rotavirus vaccines, RotaTeq from Merck, and Rotarix from GlaxoSmithKline (23). NoV is the second most important pathogen in endemic childhood infections, causing approximately 12% of severe gastroenteritis in children <5 years of age, leading to an estimated mortality number of 200,000 deaths annually (24). Sapovirus, related to NoV, has a lower incidence, and generally causes milder infections (25, 26), although the recent development of better detection tools (27, 28) is likely to increase the incidence rate. Enteric adenoviruses have a reported incidence of 1-8% in industrialized countries, whereas 2-31% has been reported in developing countries (10). Symptomatic infection of astrovirus occurs mainly in young children and the elderly, with incidences between 2.5 and 10% in children with sporadic diarrhea (29).

In contrast, looking at epidemic diarrheal disease affecting all ages, we observe a different pattern. The absolute majority of all outbreaks of gastroenteritis are caused by NoV (14), accounting for >90% of all outbreaks of viral origin, and ~50% of all-cause outbreaks worldwide (30-32).
Table 1. The most important viruses causing gastroenteritis.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Family</th>
<th>Size</th>
<th>Nucleic Acid</th>
<th>Clinical features</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rotavirus</td>
<td>Reoviridae</td>
<td>70</td>
<td>dsRNA</td>
<td>The main etiological agent of severe gastroenteritis in children less than 5 years</td>
</tr>
<tr>
<td>Norovirus</td>
<td>Caliciviridae</td>
<td>28-38</td>
<td>+ssRNA</td>
<td>Causing &gt;90% of all virus-induced outbreaks, and is second to rotavirus in endemic pediatric diarrhea</td>
</tr>
<tr>
<td>Sapovirus</td>
<td>Caliciviridae</td>
<td>28-35</td>
<td>+ssRNA</td>
<td>Generally causing milder and more infrequent infections as compared to NoV</td>
</tr>
<tr>
<td>Astrovirus</td>
<td>Astroviridae</td>
<td>28-30</td>
<td>+ssRNA</td>
<td>Primarily infects young children, many under 6 months</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>Adenoviridae</td>
<td>70-80</td>
<td>dsDNA</td>
<td>Primarily infects young children, can also give rise to respiratory symptoms</td>
</tr>
</tbody>
</table>

1.3 The emerging importance of norovirus

As early as 1929, Zahorsky first described “‘Hyperemesis hemis” or “winter vomiting disease”, an illness characterized by the sudden onset of self-limited vomiting and diarrhea that typically peaked during the winter months (33). However, the winter vomiting virus was not identified until 1972. Using immuno-electron microscopy, Kapikan and coworkers identified the NoV particles in stool of volunteers challenged with stool filtrates from an outbreak of gastroenteritis in a school in the town of Norwalk, Ohio in 1968 (34). NoV was thus originally named Norwalk virus, and is still often referred to its original name. This was also the first time a virus causing gastroenteritis was isolated. During the last two decades, there has been a dramatic increase of reported cases of NoV-induced gastroenteritis (35). This increase is partly due to the development of effective detection methods since the early years of NoV research. However, the development of more sensitive detection methods cannot account for all of this huge increase. Studies have indicated a recent emergence of highly virulent NoV strains (described more in section 2), and that variants of these strains have remained circulating, causing the high number of diarrheal cases that are now reported (36, 37). Our modern lifestyles also contribute to the increased impact of NoV. The world population is increasing with more people living in densely populated areas. NoV outbreaks
in day-care centers (38, 39), communal settings (40), nursing homes (41), and hospitals (42), are frequently reported. In addition, we eat more foods that have been handled by a number of potentially infectious people. There is a rise in the consumption of fresh vegetables and fruit, food which is often grown in countries where crops are irrigated with potentially contaminated wastewater (43). Also travelling has increased, augmenting the exposure risk at hotels, cruise ships and airplanes.

Intensive research in the last years has thus led to that NoV today is recognized as the most common cause of acute gastroenteritis in humans (30-32). The Center for Disease Control and Prevention in the United States reported that NoVs account for over 96% of all foodborne viral gastroenteritis, and over 23 million annual infections in the United States alone (44). It has furthermore been estimated that each year, NoV causes 64,000 episodes of diarrhea requiring hospitalizations, and 900,000 clinic visits among children in industrialized countries (24). Moreover, NoV is estimated to cause up to 200,000 deaths of children less than 5 years of age in developing countries (24), and other studies have indicated that NoV cause up to half of all cases of gastroenteritis world-wide, resulting in the striking number of over 267 million annual infections (45). All these infections also cause considerably economic loss, outbreaks of gastroenteritis in hospitals have been estimated to annual expenses approaching or exceeding $184 million in supplies, staff time off, and closed beds in England alone (3). These facts highlight the impact of NoV in the society, both in industrialized and developing countries, with no vaccine, effective treatments, or prevention strategies currently available.
2. Diversity and classification of norovirus

2.1 A family with many members
NoV belongs to the family Caliciviridae, a virus family in which we also find sapovirus, another virus causing gastroenteritis in humans (Table 1, Figure 2). The two other genera of the Caliciviridae family are vesivirus, including feline calicivirus causing respiratory disease in cats (46), and lagovirus, including the fatal rabbit hemorrhagic disease virus (47). NoV is one of the most diverse human viruses described, and can be divided into five different genogroups, where GGI, GGII, and GGIV cause disease in humans (48). The GGIII contains bovine strains, whereas GV contains murine strains (Figure 2) (48). The NoV genogroups exhibit a high diversity, with the amino acid sequence encoded by the capsid gene differing up to 60% (48). This high diversity makes it plausible to consider the genogroups as individual species. The genogroups can be further divided into genotypes based on sequence diversity, with at least 8 genotypes for GGI and 17 for GGII have been described (48), and a 18th genotype for GGII pending (49) (Figure 2). The most frequently occurring, and clinically severe genotype, GGII.4 (see section 3), is often further divided into specific variants to account for the recurrent seasonal pandemics (36, 50, 51).

Figure 2. The Caliciviridae family, with human pathogenic groups marked in darker grey. Norovirus is divided into five genogroups (GG), where GGI, GGII and GGIV cause disease in humans. The vast majority of human illness is caused by strains belonging to GGI and GGII, which can be divided into at least 25 different genotypes.
2.2 Structure

NoV is a non-enveloped, single-stranded positive sense RNA virus of ~7.5 kbp in length, having icosahedral capsid symmetry with a diameter of about 30 nm (21, 46, 48). The genome of NoV is organized into three open reading frames (ORF), where ORF1 encompasses ~5 kb (two-thirds of the NoV genome), and encodes a 200 kDa polyprotein, which is auto processed by a virally encoded protease to yield the non-structural viral replicase proteins essential for viral replication (Figure 3). ORF2 is ~1.8 kb in length, and encodes the 57 kDa major capsid protein VP1, whereas ORF3 is ~0.6 kb and encodes a 33 kDa small protein, VP2 whose function is unknown, but possibly plays a role in the expression and stability of VP1 (52), or with the packaging of genomes into virions. The 3’end of the genome contains a poly(A) tail (Figure 3) (53).

![Figure 3. The norovirus genome consists of three open readings frames. ORF1 encodes the non-structural proteins, with ORF2 and ORF3 encoding the structural proteins VP1, the major capsid protein, and VP2, a minor structural protein. The non-structural polyprotein of ORF1 is processed by the viral 3C-like protease (Pro), into six mature proteins: p48, an N-terminal protein of unknown function; NTPase, a nucleoside triphosphatase; p20, a protein of unknown function; VPg, which is found covalently attached to the 5’end of the viral genome; Pro, which is the 3C-like proteinase, and Pol, which is the viral RNA dependent RNA polymerase. The major capsid protein is further divided into the shell and protruding domains (P), with the P domain subsequently divided into two different subdomains, P1 and P2.](image)

The major capsid protein can be further divided into three domains, the N-terminal domain within the capsid, the intermediate shell domain, and the protruding P domain (54). The P domain is in turn divided into two major sub-domains, P1 and P2. Most of the cellular receptor interactions and immune recognition epitopes are thought to be located in the P2 sub-domain, extending above the viral surface, which is also the most variable part of the NoV genome (55, 56). The P2 domain is moreover the key domain determining host ranges of humans, with different NoV strains using different receptors (see more in section 4) (57, 58).
2.3 Detection and molecular characterization

After its discovery in the early 1970s, the only means to detect NoV was by electron microscopy (EM). This technique can visualize norovirus particles from stool, but has a high detection limit of about $10^6$ to $10^7$ viral particles per gram of stool, and requires advanced technical training in order to use (9). The inability to grow human NoVs in cell culture hindered the development of reactive antibodies, and the first immunological assays (59) had similarly low sensitivities as compared to EM. It was not until the molecular revolution with the introduction of PCR based techniques in the beginning of the 1990s (13), that the sensitivity of detection increased. With these new molecular techniques it was possible to clone and develop virus-like particles (VLPs) of NoV, which lead to more reactive antibodies for detection. With the successive accumulation of NoV sequence data over the following years, it became possible to find relatively conserved regions even for this highly diverse virus (12, 60, 61), enabling the use of broadly reactive RT-PCR assays. The next innovation came with the introduction of real-time PCR techniques for detection of NoV (11, 12, 62, 63), enabling additional sensitivity, and the ability to quantify the viruses. Real-time PCR has now emerged as standard for diagnosis and detection of noroviruses, with the immunological techniques still lagging behind in sensitivity (11). For example, two commercial immunological kits, IDEIA Norovirus from OXOID and Ridascreen from R-Biopharm, were estimated to have between 30-86% sensitivity as compared to RT-PCR methods (64-66). The antigenic diversity, and the lack of cell culture systems for NoV, will likely maintain real-time PCR as the best method for detection of NoVs in the foreseeable future.

It is possible to distinguish genogroups directly, without sequencing, using PCR or EIA based detection methodology (11, 66, 67). To determine the genotype, however, sequencing of parts of the NoV genome is required. No clear rule of thumb exists for a taxonomic assignment of norovirus genotypes. Cut-off limits for differentiating between genotypes have been suggested to 15-45% amino acid difference on the entire capsid protein, based on uncorrected pairwise distance measurements (48). If there is less than 15% amino acid difference, the strains belong to the same genotype, and if there is more than 45% amino acid difference, the strains belong to different genogroups. However, obtaining the sequence for the whole capsid is often difficult and not feasible when a large number of samples are to be investigated. Therefore, various genotype-determining approaches, based on sequencing of smaller parts of the genome, have been developed. They target different areas of the NoV genome, the RNA polymerase (68), N/S region of the capsid (49, 60), and the P domain of the capsid (69). The
first attempts to classify NoV were based on sequencing of parts of the RNA polymerase
gene, but it has lately been shown not suitable for genotyping (60), although useful for
identification of recombinant strains (70). Today, sequencing of the N/S region is emerging as
standard methodology for quick assessment of genotypes, due to its broad detection range,
and relatively high resolution (49, 60), where cut-off limits have been tentatively assigned to
22.1 ± 6.3% (GGI), and 26.1 ± 10.5% (GGII) of mean pairwise nucleotide distances in order
to distinguish between genotypes (60). Other parts in the P2 domain (region D) of the capsid
have been suggested due to its high variability (69), but these assays have a less broad
detection range than the assays targeting the N/S region (49), and are thus not suitable for
large-scale epidemiological studies. The clinical importance of the emerging GGII.4 strains
has lead to a need to further differentiate them into variants. The assignment of variants can
be based on small sequence differences, or on amino acid substitutions in specific positions of
the N/S region of the capsid (71).
3. Transmission of norovirus in the human environment

3.1 Different properties of norovirus strains

It has been observed that different NoV genotypes and genogroups have different properties regarding prevalence, transmission pathways, and host genetic factors. The most dominant NoV genotype found in most outbreaks is GGII.4, which accounts for approximately 60-90% of reported cases of NoV gastroenteritis (49, 71, 72). The pandemic outbreaks observed since the mid-90s have been due to the emergence and spread of variants of this particular genotype (36). These variants include the Camberwell cluster, which ranges from 1987 to 1995, the Grimsby cluster from 1995 to 2002, the Farmington Hills cluster from 2002 to 2004, the Hunter cluster from 2004 to 2006, and the Sakai cluster, which includes viruses isolated 2004–2006 (36, 51, 73). The reason why it is variants of GGII.4 that cause the pandemics is unclear, but studies suggest that it could be due to one or a combination of the following reasons: a higher biological fitness, larger viral shedding in infected patients (49), a more diverse receptor specificity (74, 75) (see section 4), and a fast mutation frequency. Switching human receptors, and altering antigenicity due to genetic drift would be a good way to avoid the short-term herd immunity, which is probably in place after the large pandemics (36, 76).

Except for the virulence factors and larger impact of the GGII.4 strains, NoV genotypes and genogroups exhibit differences regarding transmission pathways and seasonality (77, 78). In a large collected system review of norovirus illnesses in Europe (78), the authors found differences in transmission when stratifying disease spreading according to person-to-person, waterborne or foodborne transmissions (Figure 4).

![Figure 4. Distribution of transmission pathways between GGII.4, non-GGII and GGII non-4 NoV genotypes. Modified from (78).](image-url)
GGII.4 strains account for the absolute majority of the person-to-person spreading. This genotype is found less in foodborne and waterborne outbreaks, as compared to other GGII strains, in particular GGI strains. GGI strains account for the largest proportion of food and waterborne outbreaks. It is possible that GGI is more resistant to environmental factors than GGII (77), enhancing its long-term survival, thus causing the large proportion of food-and waterborne NoV outbreaks.

Moreover, looking at the seasonality of NoV, the large recurrent outbreaks during the winter months in Europe are mainly due to variants of the GGII.4 strains (78) (Figure 5). Then, after the winter season, GGII.4 prevalence decreases until the next winter. In the tropics, the same phenomenon has been observed, with the rain season associated with most GGII.4 induced gastroenteritis (49). GGI strains however, have a more stable and lower occurrence in clinical cases during the whole year (Figure 5). This concurs with the findings of paper III in this thesis, where we observed a stable transmission of GGI in wastewater, as compared to GGII, which exhibits a larger seasonality (77). Why the majority of clinical cases due to GGII.4 occur during the winter months is not clear, increased indoor dwelling and humidity factors are possible explanations.

Figure 5. Number of outbreaks with genotypes and variants per month in Spain, Finland, France, England and Wales, Hungary, The Netherlands, and Sweden (the subset of countries which have outbreak reports with sequence data throughout the complete period). Reprinted with permission (78).
3.2 Clinical symptoms and factors facilitating the spread of disease

The symptoms described for NoV-associated gastroenteritis include the following: nausea (79%), vomiting (69%), diarrhea (66%), abdominal cramps (30%), headache (22%), low-grade fever (37%), chills (32%) and myalgias (26%) (79). The diarrheal stool is non-bloody, lacks mucus, and may be loose or watery. In children, it appears that vomiting occurs more frequently than diarrhea, whereas in adults, diarrhea is more common (79). The average incubation period is 24–48 hours, and the symptoms typically resolve in 12–72 hours (79), although presentation of symptoms may be prolonged in some cases, particularly in the elderly, young children or the immune compromised (55, 80, 81). A study from England observed that symptoms in hospitalized persons lasted longer than for the NoV infected staff (81). Another study found that the duration of illness was longer in children < 2 years as compared to children between 2 and 5 years (7 days vs 3.5 days respectively) (80).

Furthermore, asymptomatic virus shedding occurs, and viruses have been detected up to 3 weeks after the resolution of symptoms (25, 80, 82), with even longer periods observed in children <1 year (80). This provides a large opportunity for transmission of the virus to other humans. There are also several cases of chronic NoV shedding in immunocompromised individuals (55, 83), which has been suggested to have a potential role for the evolution of new NoV strains (84).

All age groups of the human population are susceptible to NoV infection, particularly the elderly, with many outbreaks occurring in nursing homes (32, 41). In addition to increased susceptibility, the elderly often suffer from more severe disease and even death, as are the very young and the immunocompromised (80, 85, 86). NoV infections have most often been associated with consuming contaminated food or water, although spread during an outbreak is predominantly by person-to-person transmission via direct contact, exposure to aerosols, or the fecal–oral route (78). NoV outbreaks occur frequently where close human contact is difficult to avoid, such as schools, nursing homes, retirement communities, hospitals, or day care centers (38-42). There are many factors contributing to the transmission of NoV, and its ability to cause outbreaks. Firstly, it is an extremely stable particle in the environment, highly resistant to disinfectants such as chlorine, and to freezing and high temperatures (18). Furthermore, a low dose, 10 viral particles, is required for symptomatic infection (87), and prolonged asymptomatic shedding increasing the risk of secondary spread (82). Finally, the
high antigenic diversity (48), and the lack of long-term immunity (76), lead to many re-infections from the same or different NoV strains (Table 2).

Table 2: Norovirus characteristics facilitating transmission. Modified from (14).

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Observations</th>
<th>Consequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stability</td>
<td>Highly resistant to freezing, heating (up to 60°C) and disinfections such as chlorine</td>
<td>Difficult to eliminate in water, leading to infections from: oysters, bathing water, and food irrigated with sewage. Increased risk of infections in closed settings such as hospitals</td>
</tr>
<tr>
<td>Asymptomatic shedding</td>
<td>Patients can shed NoV up to three weeks after resolution of symptoms</td>
<td>Increased risk of secondary spread, is especially a problem concerning food handlers</td>
</tr>
<tr>
<td>Diversity</td>
<td>Multiple genetic, antigenic and receptor specific strains exist</td>
<td>Developed detection methods may not be sensitive for all strains. Re-infections can occur more easily</td>
</tr>
<tr>
<td>Low infectious dose</td>
<td>Less than 10 virus particles are needed for symptomatic infection</td>
<td>Increases risk of infection from person-to-person spread, droplets, secondary spread, food contamination</td>
</tr>
<tr>
<td>Lack of long-term immunity</td>
<td>Symptomatic re-infection with the same strain can occur</td>
<td>Adults are not protected although infected as children. Hinders development of effective vaccines</td>
</tr>
</tbody>
</table>

3.3 Norovirus in wastewater
The norovirus particle is highly resistant to environmental degradation, and can withstand different treatment processes (18, 88). Norovirus outbreaks resulting directly from swimming and drinking water are frequently reported (89, 90). Moreover, many countries still use sewage water to irrigate their crops, and since NoV is resistant to chemical treatment and freezing, it can contaminate all types of food, and subsequently cause disease (91). Shellfish are a common source of NoV disease. Often cultivated in water downstream a treatment plant, pathogens such as NoV bio-accumulate in the shellfish, which can lead to outbreaks if the shellfish are inadequately cleaned (92, 93). These findings highlight the importance of treatment processes that are able to reduce virus contamination, for which most wastewater treatment systems were not originally constructed. Numerous studies have shown that enteric viruses are present in high levels in water, even after the treatment process (11, 94-98). Bacterial indicators are often used to indirectly measure contamination, but they have proven
unreliable in terms of viral contamination (96, 99). There is an ongoing debate about finding a reliable viral indicator, and many enteric viruses or bacteriophages have been suggested, such as adenovirus and somatic coliphages (98, 100, 101), but no conclusions have yet been reached. Many studies on enteric viruses in wastewater usually describe virus concentrations from the influent and effluent water, but physiochemical parameters are often not considered in the investigations (97, 102, 103). This approach fails in understanding which processes of the wastewater treatment plant (WWTP) that are important for reduction of norovirus. Therefore, little is still known about factors influencing viral reduction, and how to best monitor virus contamination.

The sewage water, more than a potential health risk, can also be regarded as a mirror reflecting what goes on in the community connected to the wastewater system. By measuring the transmission of norovirus in wastewater, important epidemiological information can be obtained. For example, does the quantity of NoV in wastewater reflect the clinical picture in the community? Are there “silent” NoV shedding going on, perhaps from NoV types that cause less severe or asymptomatic symptom? Are there unknown reservoirs of NoV, and do the NoV types that circulate in wastewater relate to those found in persons with disease? Some studies from Japan and the Netherlands have observed higher concentration of both genogroups during the winter months, and that GGII is present in higher concentrations during the whole year (94, 96, 98). Another recent study found similar genotypes in wastewater as in the clinical material, suggesting that the genotypes in wastewater reflect well to the viruses circulating in the community (104). However, the understanding of noroviral transmission in the environment remains to a large extent unclear, and more studies are clearly needed to elucidate this pattern.
4. Why are some people resistant to winter vomiting disease?

4.1 A brief history of the genetic factors determining susceptibility

In the 1970s, the first isolated NoV strain detected in the Norwalk outbreak (see section 1.3), was used in a challenge study with 12 volunteers (76). Interestingly, 50% of the individuals did not develop symptoms of NoV gastroenteritis, and when challenged again 27 and 42 months later, they remained asymptomatic while the other 50% of the individuals developed symptoms at all times. A subsequent re-challenge was performed on the symptomatic individuals, this time only 4-8 weeks later, in which only one individual developed symptoms. Most symptomatic individuals had increased serum antibody titers after each challenge. Taken together, these findings indicated that there was no long-lasting immunity to NoV. A short-time immunity was noted, and other factors than serum antibodies appeared important for immunity since increased antibody titers did not offer protection. The factors mediating the resistance remained unknown for over two decades. In the beginning of the nineties, it was shown that rabbit hemorrhagic fever virus (vesivirus), also belonging to the *Caliciviridae* family, could agglutinate erythrocytes (105). Then, in 2000, it was observed that the rabbit hemorrhagic virus also had the ability to agglutinate human erythrocytes in presence of ABO antigens (blood groups) (106). Finally, it was demonstrated that the Norwalk prototype VLP bound to surface epithelial cells of the gastroduodenal junction as well as to saliva, but only to the so-called secretor positive individuals, which express histo-blood group antigens (HBGA) in saliva and mucosa (107). Thus, the investigation of NoV receptors and genetic determinants of susceptibility could begin in earnest.

4.2 Histo-blood group antigens as the probable receptors for norovirus

It is today recognized that human HBGA are receptors for NoV. Several studies (108-111) have associated NoV susceptibility to the presence of α1,2-linked fucose on HBGA, which is determined by the *FUT2* gene (112, 113). Individuals carrying at least one functional *FUT2* allele, and thus expressing the α1,2 fucosyl transferase (FucT-II) enzyme, are termed secretor-positive (secretors), and can express the A and B blood group antigens, as well as H-type 1 and Lewis b (Leb) antigens on mucosa and in secretions (Figure 6) (108-110). Individuals lacking FucT-II are termed secretor-negative (non-secretors), and have been shown to be highly protected from infections with the most common NoV genotype (GGI.4), as well as the Norwalk virus prototype strain (GGI.1) (108, 109). Saliva binding studies have
demonstrated that different NoV strains exhibit different binding patterns (74, 75, 114), with the prototype Norwalk virus (GGI.1) mainly recognizing saliva from secretors with blood group A and O, while exhibiting low or no binding to saliva from non-secretors and carriers of blood group B, all suggesting protection against infection among the latter two groups. Similarly to GGI infection, the common GGII.4 strains have been found to bind saliva from all secretors irrespective of blood group, but not to non-secretors (115). Some NoV strains such as VA207 (GGII.9), OIF (GGII.13), Boxer (GGI.8) and Kashiwa645 (GGI.3) have been shown to bind to saliva of non-secretors (74, 75), which indicates that these NoV strains also can infect the normally resistant non-secretors. However, if the binding pattern to saliva is a thorough indicator of susceptibility remains to be further investigated with authentic studies.

Figure 6. Schematic overview of the biosynthesis of ABH and Lewis histo-blood group antigens (HBGAs) by stepwise addition of monosaccharides to precursor structures. The FUT2 gene encodes an α1,2fucosyltransferase which adds a fucose residue in α1,2 linkage to the terminal galactose of the H type 1 precursor. Non-secretors, having an inactivated FUT2 enzyme, lacks α1,2-linked fucose containing HBGAs on many epithelial cells and secretions, and will not be able to synthesize the H type 1 antigen from its precursor. Synthesis of the A and B antigens requires the presence of the H type 1 antigen, adding an N-acetylgalactosamine (A) or a galactose (B) in a α1,3 linkage on the galactose residue of the H type 1 antigen. The Lewis antigens are synthesized with the FUT3 enzyme which attaches a fucose residue on the N-acetylgalcosamine of the precursor. Abbrv: Gal: galactose; GlcNAc: N-acetylgalcosamine; Fuc: fucose.
5. Aims

The general aim of this thesis project is to find ways of preventing NoV infection by studying and understanding the epidemiology of the virus. NoV is a highly diverse virus, making it difficult to develop effective detection methods. Furthermore, different NoV strains exhibit differences regarding severity of symptoms, seasonal transmission pathways, and receptor specificities in the human host. It is therefore important to detect and characterize the virus at different time-points and in different settings, enabling a fuller understanding of NoV disease. Our approach to this can be summarized as follows:

• Develop real-time PCR assays for detecting and quantifying human NoV in clinical and environmental samples

• Characterize the most predominantly circulating NoV strains and their prevalence in children’s diarrhea in Nicaragua during one year

• Elucidate the presence, seasonal variation and parameters influencing the reduction of NoV in a wastewater treatment plant during one year

• Investigate host susceptibility factors to NoV infection in a foodborne outbreak

An important aspect of all papers included in this thesis is the detection and characterization of NoV. This can be difficult since NoVs are highly variable, which makes it problematic to develop a broad detection assay able to target all the various strains. It is also important to consider the detection limit. For stool samples this is generally not a problem, since they contain high amounts of virus particles. Finding NoV in wastewater with molecular methods is much more difficult, mainly due to two reasons: First, virus in wastewater is highly diluted as compared to stool samples. Second, wastewater has a high density of other particles that can work inhibitory in RT and/or PCR reactions. This means that a very sensitive method for detection and/or a method for enriching the virus fraction in the water are warranted. We therefore established a real-time PCR assay, a method previously shown to be highly sensitive for NoV detection. For analysis of NoV in wastewater samples, we established an ultracentrifugation assay to concentrate the virus fraction before proceeding with the real-time PCR detection (Figure 7).

The real-time PCR assay was developed using fluorescently labeled primers based on the Light Upon Extension (LUX) technique. The LUX technique uses a fluorophore attached near the 3’ end of one of the primers, constructed to form a hairpin loop, and thus rendering a sterical fluorescence quenching capability (Appendix A). When the primer becomes incorporated into the double-stranded PCR product, the fluorophore is de-quenched, resulting in an increase of the fluorescence signal (116). The advantage with LUX is that it offers high sensitivity and specificity, without the use of probe or quencher molecules. This structure also hinders primer-dimer formation, which can decrease efficiency (117). The incorporation of a fluorophore moreover enables the use of melting curve analysis, which can differentiate amplicons based on sequence differences, in our case two human NoV genogroups.

Our developed real-time PCR assay was validated against stool specimens collected from both Sweden (n=61) and Nicaragua (n=42), and against a reference panel from the Swedish Center for Infectious Disease control (n=15). The same samples were also tested with other methods for comparison of sensitivity and specificity. A commercial ELISA kit (DAKO 6044), a published conventional PCR method (118), and a TaqMan real-time PCR method
modified from Kageyama and coworkers (12) were used for this comparison. Cloning of gene fragments, obtained after PCR amplification of GGI.4 and GGII.4 strains, into plasmids was performed in order to have a reference for determination of detection limits and PCR efficiency of the real-time PCR assay, and also to be able to quantify virus in stool and wastewater.

Figure 7. Flow scheme of NoV detection and quantification from stool and wastewater samples.

Analysis using bioinformatics revealed a highly conserved region in the ORF1-ORF2 junction for both GGI and GGII, which was chosen as target for the LUX primers. LUX primers were manually designed and evaluated with conventional PCR, where the best primer pairs were chosen to be used in the real-time PCR assay (Appendix A).

The real-time PCR assay was able to detect ≤10 gene copies in reference samples investigated for GGII and GGI, respectively. Ten gene copies per PCR reaction is equivalent to ~20 000 virus particles per gram of stool, or ~10 000 virus particles per liter of wastewater after being processed as described in Figure 7. These two assays were further evaluated with clinical
specimens positive for NoV GGI, NoV GGII, rotavirus, sapovirus, adenovirus, astrovirus and feline calicivirus, respectively, and no cross-reactivity was observed.

The LUX real-time PCR assay was able to detect all NoV positive specimens and assign the correct genogroup in the reference panel. We furthermore found a 99% correlation between our LUX based assay and the TaqMan real-time PCR assay with all specimens tested, with one specimen negative in the LUX assay being positive in the TaqMan assay. The LUX assay was more sensitive compared to the conventional PCR and the ELISA based methods (Table 3, paper I).

For each PCR-product a specific melting temperature interval was determined, and the melting temperature range between the genogroups was clearly distinguishable. The LUX real-time PCR assay was able to simultaneously detect and distinguish between NoV GGI and GGII positive specimens and mixed infections of these, using a duplex assay containing primers for both GGI and GGII (Figure 3, paper I). This is the first assay of its type able to distinguish between GGI and GGII based on melting curve analysis. Distinguishing between the NoV genogroups yields valuable epidemiological information, and a multiplex assay saves time and considerably lowers the screening costs.

To summarize, we developed and established a novel real-time PCR assay for detection and quantification of NoV GGI and GGII. Using specimens both from Sweden and Nicaragua, we have shown that the assays can be applied in different geographic regions, and the use of melting curve analysis can successfully distinguish between the two main human NoV genogroups. This assay can also be used to detect, quantify and assign genogroups of NoV in wastewater samples. The LUX system is simple and cost-effective, since it does not use probes or various fluorophores. The system can be used on most real-time PCR platforms, and there is no need for post PCR processing which reduces the time and possibility of contamination.
7. Characterization of norovirus in children’s diarrhea (paper II)

Earlier believed to mainly be a pathogen important in adult gastroenteritis, the big impact of NoV in children’s diarrhea is now beginning to be realized. Second only to rotavirus, NoV has an estimated prevalence of between 10-15% in all severe diarrhea episodes in children (24). Considering this, we wanted to elucidate the impact of NoV, and to characterize circulating strains in pediatric diarrhea in Nicaragua during a whole year. This is the first study of its kind in the Central American region.

The clinical specimens investigated for NoV were collected from children living in the city of León, Nicaragua. Nicaragua is located in Central America with an estimated population of 5,500,000 inhabitants; approximately 12.3% are children 1-4 years of age (119). The mortality rate in Nicaragua was 26.4 per 1000 live births between 2000 and 2005 (119), with respiratory and diarrhea illness as the leading causes of death among children 1-4 years of age (120). The climate is tropical; the rainy season starts in June, and lasts until November, when the dry season starts. Sanitary conditions are insufficient in large sections of the city of León, especially in peripheral areas.

From March 2005 to February 2006, a total of 542 children ≤ 5 years of age suffering from sporadic acute diarrhea were enrolled at five different health facilities in León, in a longitudinal prospective manner. The clinical information was obtained by reviewing the clinical records of the cases. The information was registered in a paper file containing answers to questions about symptoms such as; fever, nausea, vomiting, loss of appetite, abdominal cramps, abdominal distension (gas), number of loose stools during the past 24 hours, dehydration status and treatment plan. The disease was then classified according to dehydration status in three levels: “severe dehydration”, “some dehydration”, and “no dehydration” (Integrated Management of Childhood Illness, WHO ref WHO/FCH/CAH/01.01). Detection of norovirus was performed with commercial ELISA kits, IDEIA k6043 and 6044, and NoV positive specimens were subsequently quantified with the LUX real-time PCR (paper I), followed by genotyping by sequencing of the N/S region of the capsid gene (Figure 8, Appendix A).
Norovirus was detected in 65 (12%) of the 542 stool samples analyzed, 11% in children from the community, and 15% in a total of 133 hospitalized children. The high prevalence of NoV in hospitalized children is noteworthy, since it is more than earlier described in France, Australia and the United States (118, 121). Considering that the sensitivity of the ELISA screening assay used is less as compared to RT-PCR methods, the true impact in Nicaragua is probably 5-10% higher, indicating a very high prevalence of NoV. Surprisingly, girls (15%) were significantly more infected than boys (10%) (p=0.04), with children less than 2 years more frequently infected than children 2-5 years (Table 1, paper II). No gender specific NoV-susceptibility mutation is known, thus socio-economic factors are the likely explanation to the difference of NoV prevalence between girls and boys.

GGII was the most common genogroup observed, found in 88% (57/65) of the children, followed by GGI in 11% (7/65). The highest diversity of NoV genotypes was observed in April when at least four genotypes circulated, GGII.2, GGII.4, GGII.7 and a novel cluster, tentatively termed GGII.18. This novel cluster was confirmed by sequencing of the region D of the capsid gene (Appendix A). During June, GGL.4 and GGL.4 were observed, and in July the genotype GGL.4 variant 3 become predominant (Figures 1 and 2, paper II). During the
following months the NoV positive specimens decreased but in October, once again, GGII.4 re-appeared. In November, the uncommon genotype GGII.17 appeared, and during January 2006 the number of NoV-positive isolates increased up to 36%, which was associated with the re-emergence of GGII.4 variant 2 (Figures 1 and 2, paper II). Our observations extend previous knowledge about the emergence and selection of GGII.4 variants, and suggest that particular variants with increased fitness are selected from a pool of co-circulating strains.

Using the LUX real-time PCR (paper I), we quantified NoV shedding in children and compared the measured viral quantity to severity of symptoms, and to which genotype the child was infected with. The geometric mean viral loads of NoV GGI and GGII was $5.7 \times 10^6$ and $3.8 \times 10^7$ genome equivalents per gram of fecal specimen, respectively. Virus concentrations in specimens from children infected with NoV GGII.4 were approximately 15 fold higher as compared to those infected with other GGII genotypes ($7.2 \times 10^7$ vs. $4.8 \times 10^6$), and 13 fold higher than other GGI genotypes ($7.2 \times 10^7$ vs. $5.7 \times 10^6$). The highest viral load was observed in the group of children infected with GGII.4 and requiring intravenous rehydration (mean $3.2 \times 10^8$) (Figure III, paper II).

To summarize, we found a high impact of NoV in children’s diarrhea in Nicaragua, both in community and hospital based settings. The peak of NoV-induced diarrheal episodes were associated with variants of the GGII.4 genotype, emerging and replacing the many different genotypes circulating before the increase of diarrheal cases. Children infected with the GGII.4 genotype shed more virus as compared to children infected with other genotypes, which could provide an explanation to the high prevalence of GGII.4 in person-to-person transmissions.
8. High prevalence of norovirus in a wastewater treatment plant (paper III)

Viruses are usually not monitored in treatment plants, thus little is known about their prevalence, and which parameters that influence reduction. Furthermore, wastewater can reflect NoV shedding in the community connected to the treatment plant, where sub-clinical infections will not be observed in the clinical data. We therefore performed a NoV study at the wastewater treatment plant (WWTP) in Gothenburg, Sweden, where we sampled wastewater during a whole year (Oct 2005–Sep 2006) (Figure 9). The Gothenburg WWTP is one of the largest WWTP in the Nordic countries, receiving wastewater from nearly 830,000 person equivalents, with an average daily incoming water volume of ~350,000 m$^3$ (~4 m$^3$/s).

The WWTP is designed for biological nitrogen removal, utilizing pre-denitrification in a non-nitrifying activated sludge system, and post-nitrification in a trickling filter. During primary settling, heavy particles are removed. After the primary settling, iron sulfate is added which aggregates phosphor (Figure 9). The activated sludge contains high levels of biomass and is divided into two phases: an anaerobic phase, where the denitrification occurs, and an aerobic phase for decomposition of organic material. During secondary settling, sludge and phosphorous aggregates are removed, and the sludge is collected and pumped to the primary settling. After the secondary settling, ~50% of the water goes out into the recipient water, and the rest goes back into circulation via the nitrifying trickling filter. Sludge is extracted from the primary settlers and digested in completely mixed mesophilic anaerobic digesters with a retention time of 20-30 days. The digested sludge is centrifuged and the reject water is returned to the WWTP (Figure 9).

Our wastewater samples were taken monthly at eight different key sites in the wastewater treatment process (Figure 9), and stored at 4°C until processed with ultracentrifugation as described in Figure 7, and quantified with the developed real-time PCR assay (paper I). Physicochemical parameters were also measured in incoming and outgoing water for all sampling months, to determine their effect on viral concentrations. These measurements were performed as part of the routine at Gryaab laboratory, Ryaverket, Gothenburg, Sweden.
We found that NoV GGII exhibited higher concentration levels at all sites during the winter months, while NoV GGI exhibited higher concentration levels during the summer months. Moreover, NoV GGI exhibited smaller variation regarding virus concentrations than NoV GGII (Figures 2 and 3, paper III). The reason for the increase of GGI during summer, which was associated with a decrease of GGII, is probably due to the emergence of new GGI strains after the GGII-induced winter outbreaks.

The reduction between incoming and outgoing water was on average 1.5 log\(_{10}\) units (Table 2, paper III), which was largely the same between the two genogroups, although GGI at many times was not detected in the outgoing water, making reduction estimations uncertain. Virus concentration was reduced in the primary settling (average 0.7 log\(_{10}\) units) and in the activated sludge in combination with the secondary settling (average 0.9 log\(_{10}\) units). The trickling filter exhibited a limited reduction for the few occasions that the remaining virus was detected in the influent to the trickling filters (Table 2, paper III). The reduction averages were similar to the reduction of indicator bacteria, coliform bacteria and \textit{Escherichia coli}, which were on average 1.2 and 1.0 log\(_{10}\) units, although no correlation was observed with the viral reduction at individual sampling months. This emphasizes the importance of finding better indicators for monitoring of virus contamination.
We observed that the reduction of NoV in the WWTP varied between months, and thus related this to incoming virus concentrations, and to different physicochemical parameters. We found that higher incoming concentrations correlated to higher reductions of both genogroups, particularly NoV GGI, and that a higher inflow was associated with less reduction (Table 3, paper III). This negative correlation could be related to the fact that low flow gives less dilution and thus higher NoV concentrations, creating a higher potential for reduction. We furthermore observed that the incoming concentration of NoV GGI is significantly correlated to inflow, the less inflow the higher concentration of NoV GGI, probably due to dilution effects (Table 4, paper III). However, no such correlation exists for NoV GGII. This could be due to the fact that the presence of NoV GGII is more seasonal dependent than NoV GGI, thus disguising the effect of dilution. Levels of NoV GGII peaked during the winter months when clinical cases are more common, making it difficult to detect a decrease of concentration due to a higher inflow of wastewater. However, this is observed for NoV GGI, since it exhibited more stable concentration levels in wastewater, which could indicate that infections of NoV strains belonging to this genogroup occur at a stable rate throughout the whole year.

To summarize, we found that NoV was present in wastewater throughout the year, not only during the winter months. GGI levels increased in summer, possibly due to emerging circulations of new genotypes after the winter outbreaks. The transmission of NoV GGI was stable during the year, hence incoming concentrations was affected by dilution factors. This stable transmission in wastewater indicates that infections of this genogroup occur at a stable rate in the community, perhaps giving rise to sub-clinical or mild disease, since GGI is not frequently observed in clinical data. Primary treatment and treatment in a conventional, non-nitrifying activated sludge system reduced the NoV content by about a factor 30, and water flow and incoming virus concentration were associated with reduction.
9. New susceptibility patterns revealed in a foodborne norovirus outbreak (paper IV)

In October 2007, a NoV outbreak occurred in Jönköping, Sweden, at a seminar for health care improvement. NoV GGI was identified in the stool from some of the ill, and we understood that this would be an excellent opportunity to study host susceptibility factors to infection, since we had access to the clinical data and patient material. Moreover, GGI strains have not been studied much with regards to host susceptibility. Epidemiological investigations indicated that the lunch meal on the first day was contaminated with NoV, and subsequently the cause of the outbreak. The cook was ill four days before the outbreak started, and three days later other employees of the restaurant became ill, suggesting the restaurant employees as the probable source of NoV contamination in the food.

A total of 112 health care workers from different parts of Sweden joined the seminar. The health care workers were asked to take part of this case control study, and 83 individuals, including 4 employees from the restaurant, decided to participate in the study. In total 33 (40%) of these 83 individuals acquired acute gastroenteritis during or after the seminar. NoV disease was determined by at least one of the following symptoms: vomiting, diarrhea, or nausea combined with stomach-ache, from ~12 to 60 hours after the ingested meal. Descriptions of symptoms were obtained through a questionnaire sent out to all participants of the study. Saliva samples, for geno- and phenotyping of host susceptibility factors, were collected from all participants of the study (n=83) and stored at -20°C until further use (Figure 10). Furthermore, stool samples (n=4) were obtained from the cook, two employees, and one participant of the seminar with symptoms of NoV gastroenteritis, which enabled us to perform molecular characterization of the virus. Informed consent was received from all participants.
In contrast to earlier findings with GGII.4, GGII.3 and GGI.1 strains, we observed that 7 out of the 15 non-secretors were symptomatically infected (Table 1, paper IV), and the risk of developing symptomatic infection was approximately twice as high among non-secretors compared to secretors (Table 2, paper IV). Consistent with the secretor association, Le^{a+b} individuals had the highest susceptibility (OR [Odds Ratio] 2.42), compared to Le^{a+b} or Le^{a-b} individuals (OR 0.73 and 0.61 respectively) (Table 2, paper IV). Moreover, none of the non-secretors who were also Lewis-negative (n=3), hence lacking the Le^a and ABO antigens in saliva, were symptomatically infected. These findings indicate but do not prove that the Le^a antigen is one putative receptor for this NoV strain. The clinical symptoms were not affected by secretor status or HBGA profile (Table 3, paper IV).

We furthermore found that blood type B individuals had reduced risk of symptomatic infection of the outbreak strain (OR 0.27, p=0.11) (Table 2, paper IV). Nevertheless, 2 out of 12 individuals with blood group B developed symptomatic illness. It is possible that the α-gal in the blood type B structure partly covers an epitope needed for binding, and hence decreased the ability of the outbreak strain to infect carriers of blood type B.
Sequencing of the entire capsid gene revealed that the outbreak strain was a genotype GGL3 virus (Figure 3, paper IV). Interestingly, Shirato and coworkers (75), found that the Kashiwa645 strain, another GGL3 strain, sharing high aa homology with the outbreak strain in the P2 region (Figure 4, paper IV), bound to both secretor and non-secretor saliva to the same extent. Shirato et al. (75) also found that the Kashiwa645 strain bound to synthetic Le\(^a\) carbohydrates, but not to synthetic Le\(^b\), which is in concordance with the disease pattern in our study, with Le\(^{a+b}\) individuals having the highest OR for symptomatic infection of all HBGA investigated. Also, the Kashiwa645 strain bound weaker to B type saliva as compared to A or O type saliva. This indicates that saliva binding studies may be used as a reliable indicator of host susceptibility factors for individual NoV strains.

*FUT2* G428A genotyping revealed, for the first time to our knowledge, that heterozygous secretors were more susceptibility as compared to homozygous secretors, with twice the risk of symptomatic infection for heterozygous individuals (Table 2, paper IV). Being a heterozygous secretor may lead to lower FucT-II expression as compared to a homozygous secretor, which could increase the possibility for FucT-III (building the Lewis antigens on H-type 1 or its’ precursor) to compete with FucT-II for the H-type 1 precursor, increasing the concentration of the HBGA Le\(^a\). Possibly, there is a correlation between Le\(^a\) concentration and susceptibility to this NoV strain, since Le\(^{a+b}\) individuals had the highest susceptibility of all the HBGAs investigated in this study.

To summarize, this study describes for the first time a foodborne NoV outbreak infecting individuals irrespective of secretor status, with non-secretors and Le\(^{a+b}\) individuals having a higher risk of disease. Furthermore, we observed differences in susceptibility regarding homozygosity and heterozygosity in the *FUT2* gene, with heterozygous secretors more susceptible to symptomatic NoV infection than homozygous secretors.
10. Concluding remarks

The transmission of noroviral populations in the community and the environment is complex, and more studies need to be performed in order to understand the underlying mechanisms. However, on the first page of this concluding section, I have brought together some of the main findings from the different papers in a speculative attempt to describe the seasonal transmission of NoV strains (Figure 11). The year is divided into “high” and “low” NoV season, depending on the number of clinical cases associated with NoV.

<table>
<thead>
<tr>
<th>NoV “high season”</th>
<th>NoV “low season”</th>
</tr>
</thead>
<tbody>
<tr>
<td>Short term herd immunity to GGII.4?</td>
<td>Many different genotypes emerge (paper II)</td>
</tr>
<tr>
<td>Many clinical cases due to GGII.4 (paper II)</td>
<td>Less common strains cause disease in people resistant to GGII.4 (paper IV)</td>
</tr>
<tr>
<td>GGII.4</td>
<td>GGII.3</td>
</tr>
<tr>
<td>GGII.4</td>
<td>GGII.2</td>
</tr>
<tr>
<td>GGII.4</td>
<td>GGII.17</td>
</tr>
<tr>
<td>GGII.4</td>
<td>GGII.7</td>
</tr>
</tbody>
</table>

Figure 11: Overview of NoV transmission in humans during one year. During high season, many NoV-induced diarrheal cases are reported, which are mainly due to infections of GGII.4 strains (paper II). During this season we also observed high levels of GGII in wastewater (paper III), as determined by the LUX real-time PCR (paper I). During low season, there is less number of clinical cases attributed to NoV, and many different genotypes are found in patients (paper II). During the low season we observed high levels of GGII in wastewater (paper III). Some of these strains cause gastroenteritis in people not previously exposed in the high season, partly due to a different receptor usage than GGII.4 (paper IV).
The results from the papers included in this thesis provide valuable information, which could be used to develop preventive approaches to NoV disease. We observed that a specific genotype of NoV, GGII.4, gives rise to severe symptoms, and most of the clinical cases during the high season (paper II). This may provide a focus for development of prophylactic treatment, such as vaccines. However, as also observed in our investigations, there is a constant emergence of new GGII.4 variants (paper II), which indicates a mechanism of GGII.4 to avoid host responses, which could hinder the effectiveness of prophylactic treatments. We also found a possible explanation for the high transmission of the GGII.4 strains, since children infected with this genotype shed a higher number of virus (paper II).

The fact that ~20% of the Caucasian population is highly resistant to the GGII.4 has also been suggested to provide means for developing new treatments. By identification of functional receptors, it would be possible to develop medicines that block NoV infection. However, as we discovered in the foodborne outbreak (paper IV), the expectedly resistant individuals were the most susceptible to the more uncommon genotype, GGL3, which warrants the need for additional investigations of host genetic factors before developing such treatments. This foodborne outbreak occurred before peak of NoV-induced diarrhea, a time when according to our studies, many NoV genotypes circulate (papers II and III) before the re-emergence of GGII.4 strains, highlighting the importance of molecular characterization of the virus.

The continual occurrence of NoV in wastewater, particularly the GGI, during the whole year (paper III) indicates that infections are frequently occurring even in the low season. These variants probably give rise to more mild or asymptomatic infections, since the clinical cases are less frequent during this season. The NoV was often detected in high concentration in outgoing water (paper III), which could lead to environmental contamination in the river downstream the WWTP, and no correlation between reduction of bacteria and NoV was observed. These facts clearly demonstrate the need for improved monitoring tools for viruses to account for environmental contamination, and that a viral reduction strategy at the WWTPs needs to be implemented. The assays we have developed, with ultracentrifugation and LUX real-time PCR (papers I & III), are easy to perform and could be used for monitoring of NoV in environmental samples. In conclusion, this thesis has demonstrated the ubiquitous presence of NoV in the human environment and its high impact in diarrheal disease. The obtained insights into the epidemiology of the virus can hopefully be used towards finding preventive measures, which will reduce the number of NoV-induced gastroenteritis in the future.
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, and to Sarah, my greatest source of strength and inspiration.
References


42


Appendix A: PCR primers

<table>
<thead>
<tr>
<th>Primer pairs</th>
<th>Sequence (5'-3')</th>
<th>Position</th>
<th>Function</th>
<th>Reference</th>
</tr>
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<tr>
<td>NVG1f1b</td>
<td>CGY TGG ATG CGN TTC CAT GA</td>
<td>5291-5310</td>
<td>LUX real-time PCR for NoV GGI; ORF1-ORF2 junction</td>
<td>Nordgren et al (paper I) (11)</td>
</tr>
<tr>
<td>NVG1lux</td>
<td>gata GTG CTT AGA CGC CAT CA TC</td>
<td>5378-5360</td>
<td>ORF1-ORF2 junction</td>
<td>Nordgren et al., (paper I) (11)</td>
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<tr>
<td>NVG2flux1</td>
<td>garaa ATG TTY AGR TGG ATG AGR TTY TC</td>
<td>5012-5034</td>
<td>LUX real-time PCR for NoV GGII; Genotyping by N/S region of NoV GGI</td>
<td>Nordgren et al., (paper I) (11)</td>
</tr>
<tr>
<td>G1SR</td>
<td>CAT ACC CAR CCA TTR TAC A</td>
<td>5671-5653</td>
<td>Genotyping by N/S region of NoV GGII</td>
<td>Kojima et al., (67)</td>
</tr>
<tr>
<td>NVG2flux1</td>
<td>garaa ATG TTY AGR TGG ATG AGR TTY TC</td>
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<td>LUX real-time PCR for NoV GGII; Genotyping by N/S region of NoV GGI</td>
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<tr>
<td>Cap A</td>
<td>GGC WGT TCC CAC AGG CTT</td>
<td>6897–6914</td>
<td>Genotyping by region D in the P domain of NoV GGI</td>
<td>Vinje et al., (69)</td>
</tr>
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<td>Cap B2</td>
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<td>6738–6754</td>
<td>Genotyping by region D in the P domain of NoV GGI</td>
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</tr>
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<td>CCT TYC CAC WTC CCA YGG</td>
<td>6667–6684</td>
<td>Genotyping by region D in the P domain of NoV GGI</td>
<td>Vinje et al., (69)</td>
</tr>
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<td>Cap D3</td>
<td>TGY CTY ITI CCH CAR GAA TGG</td>
<td>6432–6452</td>
<td>Genotyping by region D in the P domain of NoV GGI</td>
<td>Vinje et al., (69)</td>
</tr>
<tr>
<td>Cap D1</td>
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<td>Genotyping by region D in the P domain of NoV GGI</td>
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<tr>
<td>290h</td>
<td>TGA CGA TTT CAT CAT CAC CAT A</td>
<td>4865-4886</td>
<td>Primers targeting the RdRp region of NoV GGI</td>
<td>Zintz et al., (118)</td>
</tr>
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<td>289i</td>
<td>TGA CGA TTT CAT CAT CAC CAT A</td>
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<td>Primers targeting the RdRp region of NoV GGI, GGII and sapovirus</td>
<td>Zintz et al., (118)</td>
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<tr>
<td>290i</td>
<td>GAT TAC TCC AGG TGG GAC TCC AC</td>
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<td>Primers targeting the RdRp region of NoV GGI, GGII and sapovirus</td>
<td>Zintz et al., (118)</td>
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<td>290i</td>
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<td>Zintz et al., (118)</td>
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<td>Primers targeting the RdRp region of NoV GGI, GGII and sapovirus</td>
<td>Zintz et al., (118)</td>
</tr>
<tr>
<td>CapGI3fw</td>
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<td>6952-6921</td>
<td>Amplification of the complete capsid gene for NoV GI, GG</td>
<td>Nordgren et al., (paper IV)</td>
</tr>
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<td>CapGI3rv</td>
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<td>6952-6921</td>
<td>Amplification of the complete capsid gene for NoV GI, GG</td>
<td>Nordgren et al., (paper IV)</td>
</tr>
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<td>428-F(biotin)</td>
<td>GAG AAG TAC CGC CAC ATC CGG GGG GAG TAC</td>
<td>403-432</td>
<td>PCR amplification and pyro-sequencing of parts of the FUT2 gene for determining secretor status</td>
<td>Bucardo et al., (122)</td>
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<tr>
<td>428-R</td>
<td>ATG GCC ACC TAC AAA GGT GCC CGG GGG GCT</td>
<td>597-568</td>
<td>PCR amplification and pyro-sequencing of parts of the FUT2 gene for determining secretor status</td>
<td>Kelly et al., (112)</td>
</tr>
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<td>Sequencing</td>
<td>GGT GGT GGT AGA AGG TC</td>
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<td>PCR amplification and pyro-sequencing of parts of the FUT2 gene for determining secretor status</td>
<td>Kindberg et al., (123)</td>
</tr>
</tbody>
</table>

a) Positions for NoV GGI correspond to reference strain Norwalk68 (M87661); positions for NoV GGII correspond to reference strain Lordsdale (X86557); positions for FUT2 corresponds to GenBank acc.no DQ32127.

b) Positions modified from the original article.