Identifying sources and transmission routes of norovirus outbreaks

Molecular epidemiological methods as the basis for targeted prevention strategies

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Voor mijn lieve Moeder en Vader

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General Introduction

Chapter 1

Background of human noroviruses

Norwalk virus, the prototype norovirus was first recognized as a cause of gastroenteritis in 1972 by means of electron microscopy (EM) on stool samples. Norwalk virus was identified by visualization of small round virus particles in stool samples from infected students and contacts during an outbreak in Norwalk, Ohio, in 1968. Originally norovirus was characterized based on the shape and structure by electron microscopic analysis. Later, immunological methods were applied however, these methods had serious limitations in accuracy and reproducibility and never provided a reliable scheme for antigenic classification of virus strains [1, 2]. In 1990, the first norovirus genome was sequenced , after which, researchers from the CDC Atlanta provided a systematic overview of the diversity of noroviruses (then named Norwalk-like virus [NLV]) based on sequencing and genetic characterization [3]. The norovirus were classified as a genus of the family *Caliciviridae* and the official name of norovirus was decided by the International Committee on Taxonomy of Viruses [4, 5]. Norovirus was originally known as the etiological agent of winter vomiting disease, and is now considered to be the most common cause of acute gastroenteritis in both developed and developing countries worldwide [6].

Taxonomy of Caliciviridae

Noroviruses belong to the *Caliciviridae* family, which is divided in 4 genera according the Class IV Baltimore scheme (*Norovirus, Sapovirus, Vesivirus, Lagovirus* [4]. Viruses of the genus *Sapovirus* are found in humans, swine, mink, and *Lagovirus* is found in rabbits and brown hares. The known host species for *Vesivirus* are sea lions, swine, cats, fish, dogs and primates and the recently identified *Nebovirus* genus is found in cattle [7]. Two additional genera have been proposed recently: *Recovirus*, detected in stool specimens of Rhesus monkeys [8] and *Valovirus* in swine [9], but these two genera have yet to be classified.

The genus *Norovirus* is divided into 5 genogroups based on whole genome sequence and similarity across the capsid gene [10, 11]. Viruses in genogroups I, II, and IV are

known to cause illness in humans, although genogroup II is also found in swine [12] and genogroup IV is also found in lions and canines [13]. Genogroups III and V viruses mainly infect bovine and murine species, respectively [12]. Genogroup II noroviruses are found in many outbreaks, whereas genogroup I noroviruses are more commonly involved in food-borne transmission, particularly in restaurants and on cruise ships [14-17]. Each genogroup is further subdivided into genotypes based on sequence similarity across the capsid gene [10, 18]. In the last years, the GII viruses, particularly the emerging GII.4 genotypes, have caused more than 90% of all outbreaks worldwide [19]. New variants of this genogroup (-type) emerge almost every other year and spread globally [20].

Genome organization

The virus particles are 28-32 nm in diameter. Noroviruses have been characterized as non-enveloped viruses with a genome consisting of a positive sense, single-stranded RNA strand of approximately 7.3-7.7 kb long. It encodes 3 open reading frames (ORFs), of which; ORF 1 is the largest (approximately 1700 amino acids) and is expressed as a nonstructural polyprotein precursor that is cleaved by the viral 3C-like protease. The proposed six non-structural proteins encoded in the norovirus ORF1 defined so far are, from N to C terminus: p48-NTPase-p22(p20)-VPg-3CLpro-RdRP [5, 21]. The RdRp region is a conserved region, and therefore is often used as a target in detection assays based on RT-PCR. ORF 2 encodes the viral capsid protein (550 amino acids) that forms the shell and protruding domains divided into the P1 and the P2 domain. The latter domain is the most variable part of the norovirus genome. In addition, the protruding domain is connected to the shell by a flexible hinge allowing for some freedom to change shape, potentially resulting in mutations in the P1/2 domains [22-26]. Expression of the ORF 2 proteins can result in self-assembled VLPs (virus like particles) with the same immunogenicity as the norovirus virions. For that reason, VLPs are often used for the development of antibody assays. The ORF 2 fragment can also be used for genotyping and phylogenetic analysis. The final ORF (ORF 3) encodes a small basic protein that has been characterized as a minor promotor protein that increases expression of ORF 2 and stabilizes the capsid [27].

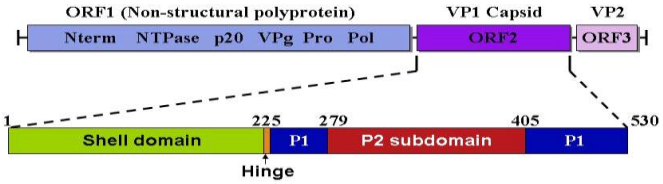

Figure 1. Schematic overview of the norovirus genome including detail representation of the ORF II, showing the most variable domain (P2 domain), (*http://emedicine.medscape.com/article/224225-overview#aw2aab6b2b2***)**

Clinical manifestations

Noroviruses are considered mucosal pathogens and are characterized often by diarrhea and or vomiting; severe symptoms like bloody diarrhea are not seen [6]. However, norovirus infections can be disruptive when they occur in healthcare institutions, and recent studies have reported that norovirus infections can lead to mortality, particularly in the elderly [28] [29]. The incubation period is short: 24-48 hours, but can last up to 72 hours whereas symptoms may last for 1-3 days. Illness may last longer in the elderly and in subjects with an immunosuppressed status [24, 30, 31]. The most commonly reported symptoms include diarrhea, vomiting, abdominal cramps and nausea and in some cases low-grade fever, chills, headache, muscle aches and fatigue. In the United States alone an estimated 23 million cases of norovirus gastroenteritis occur annually, resulting in 50.000 hospitalizations each year [32, 33]. From all those infected with norovirus, up to one-third may remain asymptomatic [34]. To some extent, the clinical burden of norovirus has been established among infected volunteers in a human challenge study, showing that at least 69 % of the challenged volunteers developed symptoms, and continued to shed the virus up to approximately 8 weeks after challenge, well after clinical recovery [35]. A commonly reported long-term consequence of norovirus infection is irritable bowel syndrome [36]. In addition, norovirus infections may lead to more severe complications in the elderly and in people with underlying illnesses: dehydration, weight loss, renal failure, disseminated intravascular coagulation (DIC), chronic diarrhea for months to years, malnutrition, and even death [6, 37]

Shedding dynamics and symptoms

Noroviruses are excreted via the fecal-oral route (via feces and vomit) with around 10^8, but up to 10^{10} RNA copies per gram stool for an average infected subject [38-40]. In contrast, an infectious dose (e.g. ID50) of few virus particles is enough to infect susceptible individuals [41]. Therefore, noroviruses are considered to have a very low infectious dose and can remain infectious for a long period of time after deposition in the environment [41]

An experimental study with Norwalk virus demonstrated that earliest onset of virus shedding was detected 18 hours post inoculation by RT-PCR and virus shedding lasted for a median of 28 days (range 13-56 days), thus demonstrating the extended intervals of shedding after recovery of subjects and the existence of asymptomatic shedding. The median peak shedding in this study was around $9.5x10^9$ genomic copies per gram stool samples as measured by the RT-PCR [35]. Individuals with symptomatic or asymptomatic norovirus infection shed virus for long periods of time, even after clinical recovery [38, 40]. The variation in numbers of viruses shed by infected subjects is considerable, ranging from 10^2 to 10^{10} viruses (qPCR detected genome copies).

The duration of shedding is also variable, with shedding in some subjects decreasing to undetectable levels within 2 weeks post infection, while in others shedding persists for up to 80 days. These factors potentially allow the virus to cause recurring outbreaks in crowded environments, like healthcare institutions, nurseries, and cruise ships. Anecdotal reports suggest that chronic shedders may play a role in sustaining the spread of infection, and also in producing new variants of the virus. The high infectivity of noroviruses and their short incubation period make them effective outbreak agents capable of causing many secondary and tertiary infections within a matter of days.

Norovirus transmission

Noroviruses can be transmitted by either direct contact (person to person), via contaminated fomites, or via food or water. The majority of the reported outbreaks are

associated with person-to-person transmission, particularly in crowded areas [20]. The virus can spread easily via droplets, aerosols of vomits, or fecal contamination, especially in absence of appropriate hygienic measures, for instance in healthcare settings and nursing homes.

Another factor of concern is the introduction of the virus into the health care setting, from the community outside. When successful, such introductions are easily spread to cause nosocomial outbreaks within days. Specific pathways contributing to the spread of the virus are usually unknown. Surveillance of norovirus in patients admitted into a university hospital (EMC) between 2002 and 2007 has revealed a considerable number of nosocomial and community-acquired cases (figure 2.) [42]

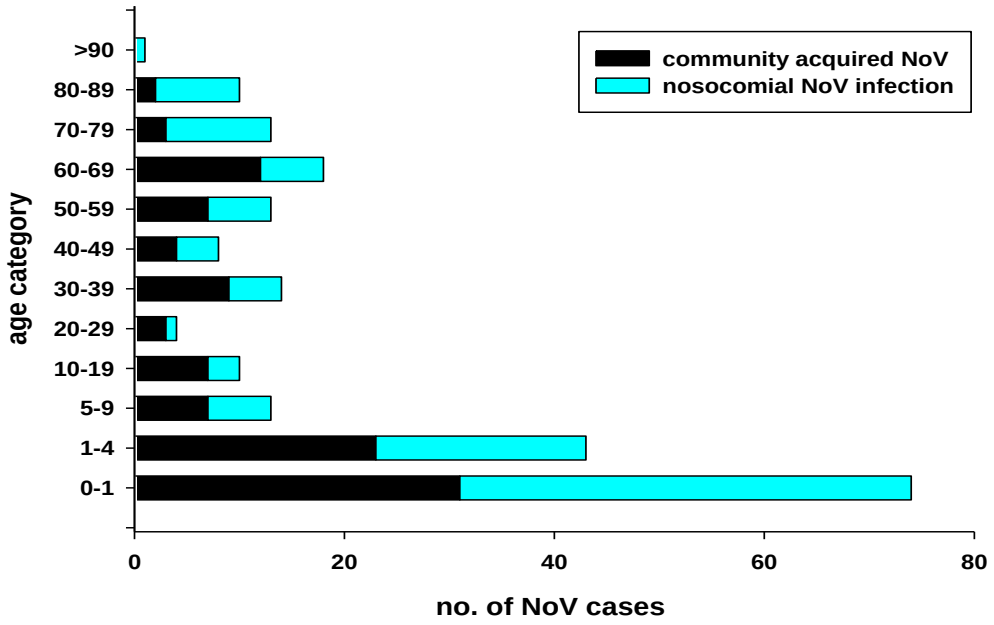

Figure 2: Number of community acquired and nosocomial infection divided among age categories over a period of 5 years (2002-07) within the Erasmus Medical Center (EMC).

Another common category of norovirus illness is food-borne outbreaks, linked to contagious food handlers or food contaminated at source (e.g. raspberries irrigated with surface water impacted by sewage). Such sources may cause outbreaks over a vast geographic region and are difficult to detect [43]. Due to the infectiousness of

these agents, any introduction may result in an outbreak within a matter of days. Understanding the environmental pathways for transmission can help improve the scientific basis for control measures. Currently, much emphasis is put on increasing levels of hygiene in institutions during norovirus outbreaks, but sources of introduction have not been studied in sufficient detail [44]. Virus deposited into the environment during outbreaks is a factor of concern since it may enhance direct, secondary and tertiary infections wherever humans come into contact with such environments. Even when a stringent hygiene protocol is enforced for HCW, hygienic measures for elderly and severely ill patients remain difficult to control.

Financial loss during outbreaks

Outbreaks caused by norovirus are costly and difficult to control, even when stringent hygiene protocols are implemented. Outbreaks do not only cause substantial health impairment in patients and health care workers, but they also lead to considerable financial losses, due to ward closure, sick leave, lost revenues, replacement of supplies and cleaning. As previously reported [45], among other common pathogens as rotavirus, influenza virus and *Clostridium*, norovirus showed the highest ward closure rate of any nosocomial infection for at least 44.1 % of the outbreaks [45]. Departments providing care for older patients are closed more often than pediatric wards, due to lack of sufficient infection control measures such as patient isolation [45]. Recent studies have provided an overview of the magnitude of financial losses during an outbreak in a tertiary care hospital in Switzerland with costs up to 40.000 USD. A similar affected hospital in the US calculated costs of at least 650.000 USD [33, 46]. In the Netherlands alone, the annual costs for norovirus at the national level were estimated to be between 33 and 69 million euros [47].

During an outbreak there is a higher than normal need for nursing care for infected individuals. Due to sick leave of HCW, affected departments may experience seriously understaffed teams with abnormally high working pressure for the remaining personnel. As a result, extra costs are incurred, for hiring extra personnel and additional financial losses due a decrease in admissions of patients or residents

[46]. On top of these costs, even more expenses may be made, for instance hiring an infection control team to contain the outbreak.

Diagnostic and detection

Worldwide, various detection methods are applied for diagnosing norovirus infection. During outbreaks, stool samples are widely used for detecting Norovirus RNA. However, other materials like vomit, food, water and environmental swabs may also be used. To date, quantitative real time RT-PCR is the most sensitive and specific assay used in many clinical and research settings, and it therefore remains the method of choice [48]. The region of the norovirus genome that is targeted by this assay is highly conserved, allowing detection of almost all genotypes and variants. Because of the use of degenerate primers, and due to the high degree of variation among noroviruses, some strains may be missed in these assays, as has been shown using cloned run-off RNA transcripts as targets (Vennema et al, unpublished). The majority of the outbreaks are dominated by GII.4 strains, and therefore the small portion of missed cases is less relevant in clinical settings [49]. Modern adaptations have resulted in improvements towards more generic and broader detection methods.

The use of quantitative RT-PCR requires sophisticated tools and highly trained personnel, which may present a barrier to implementation in laboratories around the world. Limitations of this assay are also the possible presence of inhibitors during amplification, inefficient RNA extraction, and degradation of norovirus RNA in specimens stored at suboptimal conditions.

Historically, electron microscopy was used to identify noroviruses but the sensitivity is very low (<25%) compared to RT-PCR and enzyme immunoassays [50]. An ideal norovirus detection method would be a rapid and inexpensive, yet sensitive assay that allows onsite testing. Such methods are currently on the market but are less accurate than the RT-PCR [51-53].

Despite improvements, the sensitivity of these enzyme immunoassays remains lower than that of RT-PCR [6, 54-56]. In an experimental study with Norwalk virus, detection of virus in stools could be measured by ELISA for a maximum of 10 days post inoculation, whereas RT-PCR allowed detection until at least 28 days following inoculation (ranging from 13-56 days). Therefore, exclusive use of this rapid test for

diagnosis is not recommended. However, for outbreak diagnosis this could be a valuable tool. To date, many institutions for health care, particularly nursing homes and centers for geriatric care, do not have norovirus detection assays implemented in their laboratories so that they are dependent on external testing.

Outbreak detection

The general definition of a norovirus outbreak is when at least two positive cases are found that are linked in time and place. Where norovirus diagnostic facilities are not available, outbreaks have been defined by use of the Kaplan criteria: a mean or median duration of illness between 12 and 60 hours and a mean incubation period of 24 to 48 hours, with more than 50 % of subjects vomiting, and bacterial pathogens ruled out by routine culture [57].

These criteria were based upon an in-depth analysis of 38 outbreaks of acute gastroenteritis caused by Norwalk virus between 1976 and 1980 [58]. Application of these criteria to distinguish outbreaks of acute norovirus gastroenteritis from other enteric pathogens has been associated with a sensitivity of ~70% and a specificity of up to 99 % [57, 59, 60].

Given the relative insensitivity of these symptom-based diagnostic criteria, noroviruses cannot be excluded as the etiologic agent if outbreak characteristics fail to meet the Kaplan criteria and there is suspicion for a viral enteropathogen.

In a different study, it was shown that finding a single positive sample among 2, 3 or 4 samples, using either a standard RT-PCR or a commercially available ELISA, is sufficient to identify norovirus as the causative agent in an outbreak of acute gastroenteritis [61].

Norovirus epidemiology

Since 2002, the epidemiology of norovirus appears to have changed, with the introduction of a specific new GII4 variant. Since then, continued evolution has led to the emergence of 3 successive GII4 variants and the overall incidence of outbreaks

has remained at an elevated level, compared to before 2002 [23, 49]**.** Data from outbreak surveillance have also suggested that norovirus activity has increased since the global emergence of this GII4 variant [20].

The majority of the outbreaks in health-care related institutions (nursing homes, hospitals) are associated with GII4 viruses, whereas a wider range of variants is seen in other settings such as schools, restaurants and catered events [49]. In the Netherlands, an average of 150 outbreaks is reported each year, mostly during the winter months, as well as an estimated 600.000-isolated cases of acute gastro-enteritis [62]. In addition to the increased frequency of outbreaks, an increase in the number of deaths associated with norovirus was observed in elderly, suggesting changes in the virulence of the virus [63].

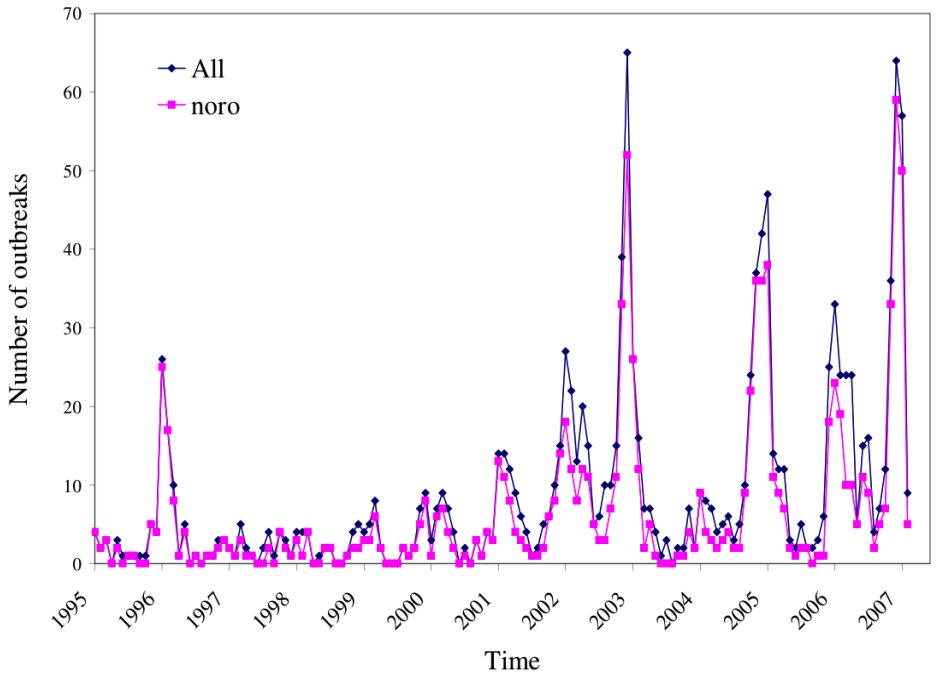

Figure 3: Number of total outbreaks reported, and number of norovirus outbreaks per month reported in the Netherlands (Source: passive surveillance of norovirus outbreaks, CIB)

These outbreaks are difficult to control because of environmental contamination: the virus tends to remain infectious outside the host, and it is relatively resistant to commonly used disinfectants. Stringent hand washing routine and the use of chlorine-based disinfectants are proven strategies to reduce transmission of

norovirus. The widespread practice of low compliance to hand washing and the replacement of hand washing by the use of (fast) alcohol-based hand rubs in hospitals considered to be effective in the combat against MRSA - may have led inadvertently to an increased risk of spread of norovirus [64]. Hand rubs have also been introduced in the food industry. Increasing the alcohol percentage to 95% appears to have some effect on reduction of contamination of a model virus (2-3 10log units), but its efficacy as an intervention in the field remains unproven. Studies on the effectiveness of different hygiene protocols are currently underway, but have shown limited success. Molecular surveillance data suggest a specific contribution of GII4 variants to the increased burden of illness in health-care settings. Therefore, understanding how these virus variants emerge and spread in hospitals and nursing homes may provide clues to improve targeting of prevention strategies

Molecular epidemiology in outbreak characterization and transmission events

Molecular sequence data of norovirus is often used for diagnostic purposes. As described in this thesis, mapping of clusters defining nosocomial transmission of norovirus can be entirely based on epidemiological information such as concurrent location and timing. However relying solely on epidemiological data ignoring any genomic sequence data can lead to misclassification of outbreaks and errors in cluster mapping.

For outbreak containment, molecular epidemiology can be used to verify links, between outbreaks and between subjects within outbreaks, providing new insights into the transmission of noroviruses. Both molecular and epidemiological data can be analyzed to unravel the transmission network during outbreaks [44, 65, 66].

Given the transmission network, the influence of preventive measures can be studied, to assess their effectiveness in limiting transmission and reducing the numbers of infections within a particular setting.

Since norovirus has the ability to mutate rapidly, understanding the rate of genetic change is essential for the design of evidence-based sequence typing for use in epidemiological studies.

Summary

To understand transmission pathways of norovirus between the general population and health care settings a standardized approach to outbreak investigations using novel methods developed and validated in an academic setting is needed. Importantly, collecting data on extent of transmission of norovirus during outbreaks, factors contributing to their introduction and spread, and data on diversity of the genomes of viruses shed by people over subsequent chains of transmission can be used to design evidence based control measures.

Scope of this Thesis

The aim of this study was to generate virological and epidemiological data on transmission of norovirus that are needed to inform policy makers on optimal targeting of prevention and intervention strategies for control of norovirus outbreaks in high risk settings. Specifically, we aimed to understand modes of transmission and sources of norovirus in outbreaks, with an emphasis on transmission within healthcare settings and between health care settings and the general population, and to assess whether norovirus infection in risk populations could serve as a reservoir for the generation of new variant norovirus strains. Finally, this thesis aims to develop evidence-based criteria for use of molecular typing data in source tracking and to provide evidence-based guidelines on options for prevention of norovirus outbreaks using the knowledge collected on transmission chains.

In **chapter 2** we evaluated the underdiagnosis of sporadic norovirus infections in a tertiary care hospital and estimated its clinical impact. From December 2008 until July 2009, fecal samples specifically referred for bacterial but not viral examination were retrospectively tested for norovirus by real-time PCR. The clinical and virological data from patients with undiagnosed (missed) norovirus infection were evaluated and compared with those from patients with recognized norovirus infection. In **chapter 3** we performed a retrospective follow-up study using a large historic data set of norovirus cases diagnosed between 2002-2007. Nosocomial transmission was re-evaluated on the basis of a combination of molecular and epidemiological cluster mapping. Admission and sampling dates were used to differentiate between patients with nosocomial infection and those who acquired their infection extramurally. Clusters were identified by sequencing the most variable P2 domain. In **chapter 4** we performed a prospective study aimed at evaluating sources and modes of transmission during norovirus outbreaks within 2 types of institutions for health care. An inclusive outbreak protocol was developed to sample all patients and healthcare workers (HCWs) with and without symptoms on wards involved in outbreaks. Additional information was collected via questionnaires provided to each participant. The epidemiological data were used to construct plausible transmission pathways and corresponding reproduction numbers for symptomatic and asymptomatic patients and

HCWs. In **chapter 5** an in-house available suite of RNA constructs for human norovirus was used to quantify the numbers of viruses shed, translating observed CT values into virus concentrations, in samples from symptomatic and asymptomatic infected subjects. In order to study the excretion of norovirus a quantitative dynamic model was fitted to the excretion data in follow-up samples of infected subjects, using a multilevel Bayesian framework to allow for individual variation in shedding kinetics. In **chapter 6** we summarized the shedding data from both symptomatic and asymptomatic subjects and compared shedding and P2 domain variation in all included patients and HCWs during all studied outbreaks. This was done to investigate genetic sequence changes in order to validate the criterion used in tracking transmission patterns that was based on 100 % sequence similarity. In **chapter 7** we review the additional data collected through responses to questionnaires, considering subject status of different types of HCW and patients and their contributions to transmission in each outbreak. We also looked at how the virus might have been introduced: types of food consumed, environmental factors such as (symptomatic) contacts or family members of infected HCW/admitted patients, with emphasis on index cases and their contribution to the spread of the virus. In **chapter 8** we described virus shedding of three chronic shedders in hospital outbreaks, showing that chronically ill patients shed virus for a long period of time and that such patients potentially act as a reservoir of norovirus transmission. Finally, in **chapter 9** we discuss the obtained results and some important mechanisms and their contribution to prevent or at least minimize future norovirus outbreaks in health care settings.

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Unrecognized norovirus infections in health care institutions and their clinical impact

Chapter 2

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Abstract

Noroviruses (NoVs) have emerged as the leading cause of acute viral gastroenteritis (GE) in humans. Although diagnostic facilities have greatly improved, significant underdiagnosis of NoV in hospitals may still occur, thereby increasing clinical burden and nosocomial spread. We evaluated the underdiagnosis of sporadic NoV infections in a tertiary care hospital and estimated its clinical impact. From December 2008 until July 2009, fecal samples specifically referred for bacterial but not viral examination were retrospectively tested for NoV by real-time PCR. The clinical and virological data from patients with undiagnosed (missed) NoV infection were evaluated and compared with those from patients with recognized NoV. During the study period, 45 patients with undiagnosed NoV were detected, whereas 50 patients had been regularly diagnosed. The missed NoV cases more frequently were adults (80% vs. 46%, $p < 0.001$). The viral load levels did not differ between the diagnosed and missed patients, but missed patients more frequently presented without diarrhea (20% vs. 4%; $p<0.07$). The newly admitted missed NoV cases with GE underwent more diagnostic imaging (24% vs. 4%; $p<0.01$) and tended to be hospitalized longer. When missed NoV patients were included, the number of nosocomial clusters doubled and missed patients were index cases in 5 of the 6 clusters. These data indicate that NoV infections are frequently missed despite routine laboratory testing and demonstrate that underdiagnosis of NoV patients is associated with costly abdominal imaging and nosocomial clustering. Awareness for NoV infection in adult patients and education about the importance of viral GE should be increased.

Keywords: norovirus; sporadic; underdiagnosis; hospital; clustering; clinical impact

Introduction

Noroviruses (NoVs) have emerged as the one of the most important pathogens causing acute gastroenteritis (GE) in children and adults (12, 8). Nursing homes and hospitals are widely confronted with NoV outbreaks. Additionally, isolated (sporadic) cases of NoV frequently occur, but their incidence and clinical impact in hospitals have not been studied systematically (2, 17). Sporadic cases of NoV may result both from community acquired infections in newly admitted patients and from nosocomial transmissions between patients, personnel or visitors (9). Although sensitive commercial and homemade diagnostic assays for NoV have become widely available, sporadic NoV infections in hospitalized patients remain underdiagnosed, increasing clinical burden and potential for nosocomial spread (1, 4, 21). Underrecognition of NoV may result in the individual patient undergoing more diagnostic procedures and may increase the influx of infectious patients into hospital wards where they may trigger outbreaks (5, 14). Apart from suboptimal laboratory facilities and inadequate specimen collection, underdiagnosis of NoV may also result from a referral bias. This bias may occur when physicians selectively refer GE patients for bacteriological or parasitological testing but not virological testing. In the present study, we prospectively evaluated underrecognition of NoV patients in a tertiary referral center with separate testing for viral and bacterial pathogens. For this purpose, the aliquots of fecal samples referred for bacteriologic testing were stored and retrospectively examined for NoV during a 6.5-month period, which included the NoV seasonal peak. The characteristics of missed NoV patients and the clinical impact on diagnosis, duration of hospitalization, and infection prevention were evaluated.

Patients and Methods

Patients

The Erasmus Medical Center (EMC) and its affiliated hospitals comprise a 1100-bed university hospital, a 269-bed children hospital, a 137-bed oncology center, and a 160-bed non-academic general hospital. From December 16, 2008 until July 1, 2009, patients from the EMC and affiliated hospitals were included in the study. Physicians

can refer patients with gastroenteritis (GE) for either bacteriological, virological, or parasitological testing, or any combination of these options. During the study period, patient samples referred for virological testing were routinely tested for NoV, whereas samples referred for bacteriological testing were aliquoted and stored at -80°C for NoV testing at a future time. Per patient, an authorized member of the medical staff accessed the following information: age, sex, date of hospitalization, results of bacteriological stool cultures, and clinical information from the virology and bacteriology database. The presence or absence of diarrhea was recorded in the laboratory. If stool samples showed no watery diarrhea, data from the medical records were reviewed to confirm the presence or absence of diarrhea. The data were anonymized with a unique code and entered into a separate database for use by the study team. The NoV RNA positive samples were stored for genotyping.

Detection of bacterial pathogens

Collected stool samples were inoculated onto McConkey (MC) agar (Difco, BBL), Salmonella-Shigella (SS) agar (Difco, BBL) and taurocholate tellurite gelatin agar (TTGA) and Brucella agar (Difco, BBL) supplemented with 5% sheep's blood and five antibiotics (amphotericin B, cephalothin, polymyxin B, trimethoprim, vancomycin) for the isolation of Salmonella, Shigella, Vibrio and Campylobacter spp. respectively. All the plates were incubated at 37°C for 18–24 h except for Brucella agar, which was incubated at 42°C in an anaerobic jar with a CampyGen pack (CN0025, Oxoid Ltd, UK) for 48 h. Along with direct streaking, each sample was enriched in selenite broth (Difco, BBL) and bile peptone broth at 37°C for 18–24 h to enhance the isolation of Salmonella spp. and Vibrio spp., respectively. The enrichment broth for Salmonella was subcultured onto SS agar and the enrichment broth for Vibrio was subcultured onto TTGA agar and incubated at 37°C for 18–24 h. Bacterial enteric pathogens were identified by colony characteristics, and by biochemical tests using conventional and API 20 biochemical profiles (bioMérieux, France) when necessary. Isolates were further confirmed serologically using commercially available specific antisera (Denka Seiken, Japan). Campylobacter spp. isolates were differentiated as C. jejuni and C. coli by the hippurate hydrolysis test. Cefsulodin-irgasan-novobiocin (CIN) agar was planted for isolation of *Yersinia*

enterocolitica. Faeces were evaluated for *Clostridium difficile* toxin by ImmunoCard® Toxin A and B (Meridian Bioscience).

Detection of norovirus by Real Time PCR

Two hundred µg feces (200 µl if liquid) were suspended in 600 µl star buffer that had been preheated in a 37°C water bath. Each tube was vortexed briefly, and 80 µl of chloroform was added. After vortexing, samples were clarified by centrifugation for 1 minute at maximum speed (Eppendorf 4515 R). A 190 µl aliquot of supernatant and 10 µl of an internal control were transferred to the Magna Pure LC Isolation plate for RT-PCR (program: total nucleic acid extraction according to the manufacturer's instruction; MagNa Pure LC) with an elution volume of 50 µl (Roche Diagnostics GmbH). For detection, 20 µl RNA extractions were reverse transcribed to cDNA with random hexamers using the MultiScribe™ Reverse Transcriptase kit (Applied Biosystems) according to the manufacturer's instructions. Subsequently, the cDNA was used in a Real-Time NoV PCR assay for qualitative analysis (2, 11).

Molecular analysis of norovirus

cDNA was amplified by a semi-nested PCR and subsequently region A of the polymerase gene was sequenced using the ABI-PrismBigDye Terminator v3.0 Ready Reaction Cycle kit (ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) as described (20). Sequences were assembled in bionumerics (Applied Maths, Sint-Martens-Latem, Belgium, software package 6.6.4), evaluated manually for their quality by looking for the number of ambiguities, errors, mismatches and deletions , and genotyped with an NoV-genotyping tool (URL: http://www.rivm.nl/mpf/norovirus/typingtool)(13). Phylogenetic analysis (UPGMA, Multiple alignment) was done to identify links between strains for each genotype (variant) separately.

Nosocomial transmission

Given an incubation period for NoV of one to three days, community acquired NoV infection was assumed if stool had been sampled in relation to GE complaints and within one day of admission. Nosocomial transmission of NoV was assumed if patients had been sampled for the first time more than 5 days after admission, as described (2). From the patients sampled on days 2-5 after admission, clinical information about the presence of GE symptoms at the first day of admission was used to differentiate between nosocomial and community acquired NoV infection. Clustering of NoV infections was defined as the presence of 2 or more patients with NoV in one ward within 5 days after the onset of disease, with at least one nosocomially infected patient (2). In addition, the patients had to be part of a molecular cluster, A molecular cluster was assumed if strain sequences were identical (for a 200bp region A pol gene fragment) or had maximally 1 mismatch over a 600bp fragment of the polymerase gene . This approach was validated by comparison with strain sequences entered into the noronet database around the same period of time from other parts of the country as well as internationally. Only the admitted patients were included in the cluster analyses.

Statistical analyses

The clinical and laboratory data from patients initially suspected of and diagnosed with an NoV infection (recognized NoV patients) and those of patients suspected for bacterial GE with an undiagnosed NoV infection (missed patients) were compared using the SPSS statistical software package (version 15; SPSS, Chicago, IL, USA) and SAS (version 9.2 for Windows; SAS Institute Inc., USA). The Mann-Whitney U test (two-tailed) was used to compare the average length of stay in the hospital for recognized NoV patients and missed patients. P values <0.05 were considered statistically significant. Logistic regression analysis was performed to determine which of 19 variables (Table 1) could be identified as univariate predictors of a missed NoV infection. Those variables with a p-value of less than 0.20 in the univariate analysis were included in the multivariate model. The variables remained in the multivariate model if the p value was less than 0.10, whereas the backward selection procedure was used. The missing values were classified as unknowns so that

the maximum number of cases was included in the multivariate logistic regression model. The analyzed variables were included as continuous variables where possible or categorized based on 50-percentiles in the group of recognized NoV cases.

Table 1. Characteristics and clinical parameters of recognized and missed NoV patients in a tertiary care hospital from 16 December 2008 to 30 June 2009

Parameter[a]	Value for undiagnosed patiënts (N=45)		Value for diagnosed patiënts (n=50) .		Univariate analysis[c] OR	95% CI
Gender male	58	26/45	40	20/50	ns	
Mean age (years)		42		31		
Age in categories: ≥18	80	36/45	46	23/50	**4.7**	(1.87-11.8)
Outpatient	21	10/45)	10	(6/50)	ns	(0.69-6.33)
Admitted with comm. acq. GE	40	(18/45	40	(20/50)	ns	
Admitted without GE symptoms #	38	(17/45)	49	(24/50)	ns	(0.29-1.49)
Pre-existing disease(s)	53	(24/45)	63	(30/46)	ns	
Immunocompromised	31	(14/45)	43	(21/50)	ns	
Chronic NoV	4	(2/45)	2	(1/50)	ns	
Vomiting	42	(13/31)	50	(19/38)	ns	
Diarrhea	80	(28/35)	95	(39/41)	0.21	(0.04-1.06)
Abdominal imaging	24	(11/45)	4	(2/50)	**7.76**	(1.62-37.3)
Mean Ct value (cycles)		24,4		24,6		
Illness duration at diagnosis (days)	4.7 days	n=28	3.8 days	n=39	ns*	
Admission duration total (days)	16.3 days	n=33	18.1 days	n=45	ns*	
Admission comm. acq. (days)	6.2 days	n=17	4.7 days	n=20	ns*	
Clustering (patients)	15	3x (2.2.2)	16	3x (3.2.2)		
Mortality 1 month	2	(1/45)	2	(1/50)	ns	
Mortality 1 year	9	(4/45)	6	(3/50)	ns	

[a] Comm. -acq., community-acquired
[b] Nosocomially infected and/or asymptomatic patients
[c] Values in boldface are statistically significant. 95% CI, 95 % confidence interval; NS, nonsignificant; *, Mann Whitney U test (two-tailed).

Results

Samples and patients

From December 2008 until July 2009, 1809 patients were tested in the departments of virology (606 patients) and bacteriology (1203 patients) of the EMC. In the virology department, 50 patients (8%) tested positive for NoV (called "recognized NoV patients"). Among the patients submitted for bacteriological testing, our retrospective analysis revealed 45 (4%) additional NoV patients ("missed patients"), which had not been diagnosed in the virology department (Fig.1). For all patients combined, the diagnostic yield was 5.3% (95/1809).

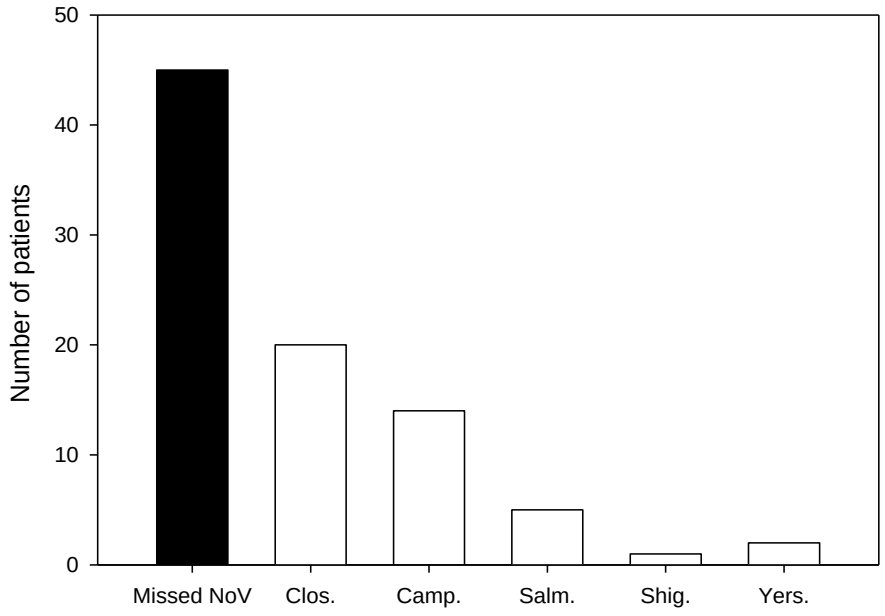

Figure 1. Missed norovirus infections in relation to bacterial infections in stool samples of patients (n=1203) sent for bacteriological culture during Dec. 2008 – July 2009. Clos.: *Clostridium difficile*; Camp.: *Campylobacter* spp; Salm.: *Salmonella* spp; Shig.: *Shigella* spp; Yers.: *Yersinia* spp.

Characteristics and clinical symptoms of recognized and missed patients

The characteristics of the recognized (n=50) and missed (n=45) NoV patients are shown in Table 1. When compared to the recognized patients, missed patients more frequently were adult (age≥18) (80% vs. 46%; p<0.01), male (58% vs. 40%; p=0.1) or outpatient (21% vs. 10%; p=0.27). A substantial number of NoV patients in both groups suffered from underlying diseases (51% vs. 63%) or were immunocompromised (31% vs. 42%), but these differences were not significant. Missed patients with NoV infection were more frequently from the affiliated non-university hospital (24% vs. 6%; p<0.02) but not more frequently related to a specific ward or department. Most of the clinical and virological characteristics were similar for recognized and missed NoV cases. These characteristics included viral load levels in fecal samples, reflected by a mean cycle threshold (Ct) of 24 for both groups, and the presence of vomiting (42 vs. 50%). However, diarrhea was less commonly reported for the missed patients (80% vs. 95%; p=0.07). Most patients not reporting diarrhea (n=9) were adults with complex underlying conditions, such as cancer liquid feeding, or end-stage diseases. In two other patients, stool samples had been taken in the context of a bacteriological screening protocol. Fot both missed en recognized patients, the patients without diarrhea had significantly more often high Ct values (> 25) compared to the patients with diarrhea (6/2 vs 24/43; OR 5,4 p<0,05).

Factors associated with missed NoV cases

In logistic regression analysis, 9 of the 19 investigated factors were associated with being a missed NoV case (Table 2). In multivariate analysis, the following factors were independently associated with being a missed case: abdominal examination and admission to the affiliated general (non-academic) hospital. The factors identified to be independent indicators of recognized cases were admission at the children's hospital, symptoms of diarrhea and higher age (in years). The effect of risk of higher age is no longer present after correcting for hospital departments.

Table 2. Risk ratios and 95% confidence intervals for independent associations between different variables and missed norovirus cases[a]

Variable	Category		Univariate analysis		Multivariate analysis	
		No. of	OR	95% CI	OR	95% CI
Gender	Female	49	Ref		Ref	
	Male	46	2,1	(0.9-4.7)	ns	
Age in years	continuous	90	**1,01**	(1.00-1.03)	**0,95**	(0.91-0.98)
Age in categories	<18	36	Ref			
	>=18	59	**4,7**	(1.9-11.8)		
Hospital visit due to acute GE (5 not diagnosed)	No	45	Ref		Ref	
	Yes	45	0,4	(0.1-1.5)	ns	
Admission duration (1 not diagnosed)	0 (outpatients)	16	Ref		Ref	
	1-9 days	38	0,5	(0.1-1.7)	ns	
	>9 days	40	0,3	(0.1-1.0)	ns	
Group	University adults	45	Ref		Ref	
	University children	36	**0,3**	(0.1-0.7)	**0**	(0.0-0.2)
	other	14	2,9	(0.7-12.0)	**5,2**	(1.0-27.7)
Symptoms of acute GE (18 missing)	No	7	Ref		Ref	
	Yes	70	0,3	(0.1-1.7)	ns	
Diarrhea (19 not diagnosed)	No	9	Ref		Ref	
	Yes	67	0,2	(0.0-1.1)	**0,1**	(0.0-0.8)
Abdominal examination	No	82	Ref		Ref	
	Yes	13	**4,5**	(1.1-17.5)	**11,7**	(2.3-58.0)
Kidney failure	No	82	Ref		Ref	
	Yes	13	0,3	(0.1-1.1)	ns	

[a]Patients were in a hospital population from 16 December 2008 to 30 June 2009. Values are from univariate and multivariate logistic regression analysis. Boldface indicates statistically significant difference. Ref, reference category; NS, not significant.

Hospitalization and diagnostic imaging

Twenty-eight missed patients (10 outpatients and 18 newly admitted patients) had been infected outside the hospital (community acquired NoV). The presumptive diagnosis for these patients at presentation is shown in Table 3. For 25 (86%) of the patients, the presumptive clinical diagnosis was not confirmed, and NoV infection was the likely explanation for the clinical symptoms in all of these cases. In two patients, the presumptive diagnosis was confirmed. However NoV infection was clinically relevant in one of these patients.

A total of 11 missed patients underwent abdominal imaging (including abdominal echo, X-ray, computerized tomography (CT) scan or duodenal/colonoscopy) during their diagnostic work-up for acute GE. When compared to the recognized patients, abdominal imaging occurred significantly more often in the missed patients (24% vs. 4%; $p<0.01$) (Table 2 and 3). Most (8 out of 11) of the missed patients with diagnostic imaging had underlying diseases. The imaging in these patients usually was performed to exclude exacerbation, complications, or progression of these underlying diseases, although in one patient the imaging was part of a routine control. In all three patients without underlying disease the diagnostic imaging was performed to explain abdominal complaints that potentially might have been due to NoV infection.

Finally, duration of hospitalization was compared between the newly admitted recognized (n=17) and missed patients with NoV (n=20). Overall, the duration of hospital stay for those with the community acquired NoV tended to be longer for the missed patients (6.2 days vs. 4.7 days) but the difference did not reach statistical significance (Mann Whitney u test: $p=0.48$).

Nosocomial spread and clustering

We evaluated nosocomial clustering in the recognized and missed hospitalized NoV patients. For this analysis we excluded the outpatients. Apart from clustering in time and place, genotyping was performed to link the cases within the hospital as described in the method section. When only the recognized patients were considered, three clusters consisting of 2 patients each were present. When missed NoV patients were included, 3 more clusters of 3, 2, and 2 patients each would have been recognized. Furthermore, two of the previous clusters would have increased with 3 and 1 patients, respectively, and one cluster would have been identified 4 days earlier. Based on the onset of disease symptoms, missed patients were designated as index cases in 5 of the 6 clusters.

Discussion

The present study was initiated to assess the potential underdetection of NoV as a cause of illness in patients admitted to the hospital or during hospital stay by retrospectively analyzing stool samples that were sent to the laboratory to exclude bacterial causes of intestinal complaints. We found that this approach approximately doubled the number of recognized NoV shedders and that the missed NoV patients underwent significantly more diagnostic imaging for GE, including colonoscopies, computed tomography, and X-ray examinations. This underrecognition of NoV mainly originated from inadequate referral to the laboratory by clinicians and therefore occurred regardless of the availability of a routine diagnostic NoV RT-PCR offered on a daily basis. To our knowledge, this routine is not atypical, and therefore, similar rates of underdiagnosis may occur in many hospitals (10, 21). We demonstrated not only that patients with unrecognized NoV infection had significantly more costly additional non-laboratory procedures but were also most likely sources for nosocomial infection in 5 instances during the relatively short period of time evaluated. Therefore, the results are relevant not only for individual patients but also for hospital infection control and for tracing NoV transmission chains.

Underdiagnosis of NoV occurred significantly more frequently in adults (60%) than in children (26%). However, this difference was no longer present after correcting for hospital departments, which suggests that the increased risk for underdiagnosis in adults likely relates to a low awareness of viral GE among physicians in adult wards rather than the patient's age. Alternatively, the general awareness for rotavirus in children might contribute to the effective recognition of viral GE including NoV infections in children.

Several recent studies underscore that NoV infection affects people of all ages and can cause severe disease in elderly and immunosuppressed individuals (3, 4, 10, 15, 18, 19). In our study, the relevance of NoV infections in adults was emphasized by the finding that NoV infections in adults largely exceeded the number bacterial GE infections based on the currently used methods for detection (59 vs. 36 patients).

The clinical characteristics and mean viral loads of the missed NoV patients were comparable to those of the recognized patients. This indicates that missed patients were not predominantly patients with a mild or late stage of disease or with a low

viral load. The only exception on this was the finding that significantly more missed NoV patients reported an absence of diarrhea when compared to diagnosed patients (14% versus 5%). However, the absolute number of missed NoV patients without reporting diarrhea was low, and most of these patients had complex underlying diseases for which diarrhea may not have been reported explicitly.

Table 3. Presumptive diagnosis and abdominal imaging results for missed NoV patients presenting at the emergency room with community-acquired NoV infection[a].

Presumptive diagnosis	Abdominal imaging	Diagnosis confirmed[b]	NoV relevant[c]
Cholecystitis	-	+	-
Subacute bacterial peritonitis	Sigm.scopy + echo	+	+
Aids related pneumonia	-	-	+
Inflammatory bowel disease	echo + colonoscopy [d]	-	+
***C. difficile*, exacerbation CU**	X-ray + Sigm.scopy	-	+
Protein-losing diarrhea.	X-ray + scopy (2x) + CT .	-	+
Addison crisis	-	-	+
Food-poisoning	X-ray	-	+
Renal dysfunction (n=3)	-	-	+
Thymoma, Giardia, CMV	Endoscopy + CT.	-	+
Graft versus Host Disease	-	-	+
Coeliac disease, CU, M.Crohn.	Echo	-	+
Diverticulitis	Sigm. scopy	-	+
AIDS presenting symptom	-	-	+
Ileus	X-ray	-	+
Tropical infection (n=2)	-	-	+
Bacterial infection (n=10)	-	-	+

[a] Twenty-eight NoV patients presented at the emergency room with community-acquired NoV infection. CU, colitis ulcerosa; CT, computed tomography; CMV, cytomegalovirus; echo, abdominal echo.
[b] +, presumptive diagnosis confirmed
[c] +, NoV infection retrospectively explained the clinical symptoms
[d] Routinely tested.

Comparing the clinical parameters between missed and recognized NoV patients, our study highlights the clinical impact of missing NoV infections within the hospital setting. First, the results demonstrate that missed NoV patients were involved in 5 out of 6 nosocomial clusters that occurred during the study period. Excluding the missed NoV patients, only three such clusters would have been recognized, one of which would have been at a later point in time. In all five clusters, the index was found to be a missed patient, which suggests that diagnosing these missed patients could

effectively improve the timely institution of infection preventive measures. Friesema et al. recently reported a beneficial effect of the early institution of preventive measures for NoV. (7). Second, we found that missed NoV patients underwent significantly more abdominal imaging than recognized patients, including colonoscopy, computed tomography, and abdominal X-ray examination. These investigations usually were requested in relation to abdominal complaints (3 patients), but also to exclude exacerbations and complications of preexisting conditions (8 patients). Our findings that most (9 out of 11) imaging remained negative and that the recognized patients had similar underlying diseases but significant less imaging, support the view that physicians may request less diagnostic imaging when norovirus is diagnosed. In this context it should be stressed that the mere presence of norovirus should not always exclude other causes of GE, since the infection can be asymptomatic in patients, especially when the viral load is low. Third, a subgroup analysis of the newly admitted patients with community-acquired GE showed that hospitalization tended to be longer for the missed NoV patients when compared to recognized patients. Although not statistically significant ($p<.0.1$), this difference may indicate that the laboratory diagnosis of NoV enables a more rapid discharge of newly admitted patients with GE.

Although we assumed that hospitalized patients with symptomatic GE were routinely sampled to eliminate an infectious cause, it is possible that in a small number of patients, notably those patients with only mild or no symptoms, no sampling was performed. Consequently, the underascertainment of NoV patients might be even higher than we report here. Furthermore, we did not address undiagnosed NoV infections among hospital personnel, although recent studies have indicated that infected personnel can play an important role in the NoV transmission chain (16). Hence, appropriate collection and testing in both patients and personnel will be required for developing new evidence-based strategies to prevent the introduction and spread of NoV (20). The presented data demonstrate that a substantial level of underdiagnosis of NoV may occur in hospital settings and stress the need for education about the importance of viral GE to physicians in these settings. Since our results confirm that missed NoV patients are associated with increased clinical burden and nosocomial clustering, routine testing for NoV in adult patients with GE during the NoV seasonal peak likely will be cost-effective (6).

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Using molecular epidemiology to trace transmission events of nosocomial norovirus infection

Chapter 3

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Abstract

Background. Nosocomial norovirus (NoV) infection is common and may lead to complications in vulnerable hospitalized patients. Understanding sources and modes of transmission of noroviruses within health care settings will support the design of evidence-based strategies for reducing introduction and further spread.

Methods. We sequenced a highly variable segment of the genome to identify possible clusters, in patients with and without acute gastroenteritis hospitalized from 2002-2007. Admission and sampling date were used to separate patients with nosocomial infection from those who were not nosocomial.

Results. Epidemiological clustering retrieved 22 clusters defined as ≥2 nosocomially infected patients on the same ward within five days. In total, 264 patients (out of 2458 tested) were diagnosed with NoV, and 61 % of the patient strains could be genotyped. Of those, 51 % (n = 82) belonged to GII.4, 34% (n = 54) to GII.3, and 15 % (n = 24) belonged to other genotypes (GI.6B, GII.17, GII.7, and GII.2). In children's wards, GII.3 strains were more often associated with nosocomial spread than other viruses, whereas in adults this was the case for GII.4 strains. Sequence alignment recognized eleven new clusters based on identical P2 domains (4 GII.3 and 7 GII.4), involving patients in different wards. This increased the total number of recognized clusters by 50 %.

Conclusion. Five of these clusters involved at least one out-patient, providing a possible target for improvement of infection control. We conclude that the use of sequence-based typing should be considered for identifying hidden nosocomial clusters of NoV infections within health care settings.

Introduction

Noroviruses (NoV) belong to the family *Caliciviridae,* and are the most common cause of acute viral gastroenteritis worldwide (11). Noroviruses have a positive sense RNA genome with an average length of 7.5 kb (4, 8). Noroviruses are genetically highly variable and are classified into 5 genogroups (GI, GII, GIII, GIV and GV), three of which are found in humans (12, 24).

NoV are usually transmitted from person to person, but may also spread via contaminated surfaces, food and water (17). NoV outbreaks are common, particularly affecting health care institutions such as nursing homes and hospitals, but their impact and modes of transmission have not been assessed systematically (1, 6, 18). Previously, we described a high frequency of nosocomially acquired infections by comparing time of diagnosis and date of hospitalisation of newly diagnosed patients (1). There is evidence for increased health expenditures and possible complications in high risk patients of nosocomial norovirus infections, showing that studies are required to develop effective methods for reducing nosocomial infections (23). A study examining the efficacy of control strategies found that implementation within 3 days after the first cases was the only factor that significantly reduced the size and duration of NoV outbreaks in nursing homes, regardless of the infection control protocol that was followed (5). Furthermore another study monitoring gastroenteritis outbreaks in England demonstrated the potential effectiveness of ward closure in hospitals (15). This shows that timely detection of nosocomial spread is a key determinant of successful control activities (7, 9, 21). We therefore investigated the possible use of molecular typing in addition to routine monitoring for nosocomial infections to detect transmission pathways of norovirus in a hospital environment. Sequencing of the norovirus P2 domain, which is located in the ORF 2 capsid gene, was used to link patients with identical strains into clusters (24, 25). This approach identified possible clusters that would be missed by standard epidemiological cluster analysis.

Materials and Methods

Data collection and fecal specimens

Data on norovirus positive cases diagnosed between 2002 and 2007 were retrieved from the database of the hospital laboratory and grouped as nosocomial cases, outpatient cases and community acquired cases (1). We used a conservative estimate to ensure high specificity by considering the possibility of nosocomial transmission only if a patient was diagnosed with NoV infection for the first time > 4 days after admission. Patients tested positive for NoV 0-1 days after admission were defined as community acquired cases. Patients with NoV positive stools diagnosed 2-4 days after hospitalization were classified as indeterminate. On the basis of the > 4 day cut-off, 22 nosocomial clusters had previously been obtained using epidemiological criteria (defined as ≥2 nosocomially infected patients with NoV on the same ward within 5 days) (1). Background data listing age, sex of patient, ward where the patient was hospitalised, date of hospitalisation, and date of onset of diarrhoea were drawn from the hospital database. This extraction was done by an authorised person, who also anonymized the records prior to use by the research team, in compliance with regulations on use of patient data.

Outline

Stored fecal specimens (stored at -80°C) were retrieved, viral RNA was extracted and strains were typed using a two-step approach: first, viruses were assigned to a genotype by sequencing region A of the polymerase gene (22). Subsequently the corresponding P2 domains in the capsid gene were sequenced, with a specific P2 primer set for each genotype (24). This approach was necessary because the genetic diversity of noroviruses P2 regions is so high that a single set of primers has inherent low sensitivity.

RNA Extraction and RT-Reaction

Faecal samples were suspended (200 mg/ 200 µl in Hanks' medium (800 µl) containing penicillin, and clarified for 30 minutes at 3000 rpm/ 4°C (8000 rcf, eppendorf 4515 R) 200 µl of the supernatant was transferred to the Magna Pure LC plate for RT-PCR (program: total nucleic acid extraction, according to the manufacturer's instruction; MagNaPureLC) with an elution volume of 50 µl (Roche Diagnostics GmbH). For genotyping, 20 µl RNA extract was reverse transcribed to cDNA with random hexamers using the MultiScribe™ Reverse Transcriptase kit (Applied Biosystems) according to the manufacturer's instructions. All the obtained CT values in our study correspond with the Real-Time PCR as previously mentioned (1).

Genotyping and P2 domain sequencing

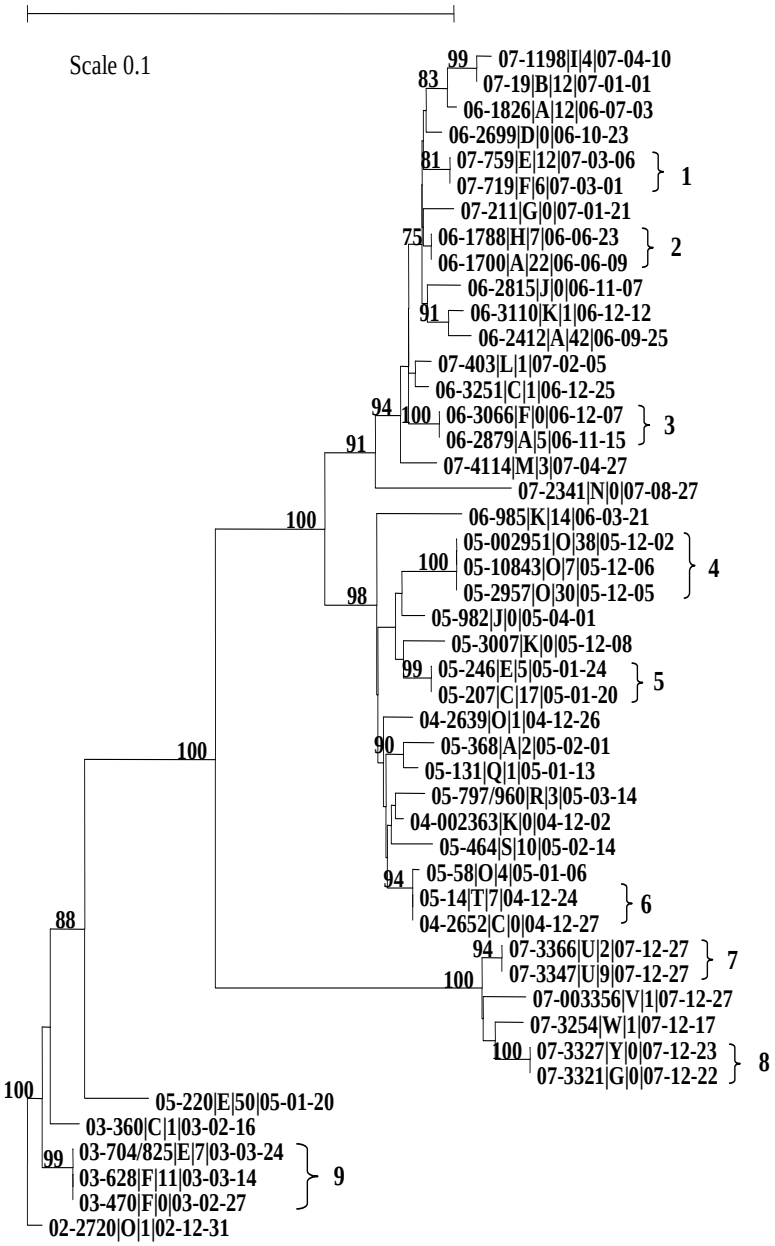
A semi-nested PCR was performed on the polymerase region (region A) to type the NoV strains. First round PCR reactions were performed with the primer set FW-JV12 (ATACCACTATGATGCAGATTA) and COGREV (TCGACGCCATCTTCATTCACA)) (10), 10 µl cDNA was added to 40 µl mix/ reaction, containing 5 µl PCR buffer, 1 µl FW/ REV primer (70 pmol), 2 µl MgCl2, 29.5 µl Adest, 1 µl DNTP's (10 mM), 0.5 µl Hotstart Taq polymerase (2 Units). The 2nd round PCR reaction (semi nested) was performed with primers set JV12-FW and JV15-REV (CTCATCCAYCTRAACATNGNYTCYTG). Two µl of the 1st PCR product was added to 48 µl mix/ reaction, containing 5 µl PCR buffer, 1 µl FW/ REV primer (70 pmol), 2 µl MgCl2, 37.5 µl Adest, 1 µl DNTP's (10 mM), 0.5 µl Hotstart Taq polymerase (2 Units). Both PCR's were performed using Gene Amp 9700 (Applied biosystems, USA) with the following cycling conditions: 96 °C for 15 min, (1 cycle) 96 °C, 52 °C, 72 °C each 1 min for (40cycles), 72 °C for 10 min). PCR products were loaded on 2% agarose gels (stained with syber safe). When the target band was observed (approx 650 bp), the PCR products were purified with ExoSAP-IT (USB corporation, USA Cleveland Ohio) (2 µl ExoSAP-IT - 5 µl PCR product) followed by sequencing with the same primers (used for PCR) using ABI Prism BigDye Terminator 3.1 approach (Applied Biosystem 3730 DNA Analyzer); denaturation 96°C for 10 sec, 96 °C -10 sec, 50 °C -5 sec and 60 °C- 4 min (25 cycles). After assignment of genotype, type specific primers were used to sequence a 794-818 nt target covering the P2 domain (24). This was done only for the two most common

genotypes GII.3 and GII.4 for which sufficient background data was available for sequence similarity comparisons. Primers and PCR conditions were as described (24).

Sequence analysis

The obtained sequences were entered and aligned in Bionumerics (software package 5.1, Applied Maths), and typed with a genotyping tool for noroviruses (URL: http://www.rivm.nl/mpf/norovirus/typingtool). The sequences were connected with the available background data file listing age, sampling date, date of discharge from the hospital and location (ward) where the patient stayed while hospitalized. (19). The P2 domain sequences with an average length of nt 550 were compared using the Neighbor Joining method (TREECON for Windows) to identify patients that had identical sequences in order to create molecular clustering. Sets of identical sequences were defined as clusters (Figure 1). The community acquired cases served not only as background sequence data in the comparison but were also used to link with nosocomial cases in order to detect introduction of strains into the hospital.

A)GII.4


Scale 0.1
07-1198|I|4|07-04-10
07-19|B|12|07-01-01
06-1826|A|12|06-07-03
06-2699|D|0|06-10-23
07-759|E|12|07-03-06
07-719|F|6|07-03-01
1
07-211|G|0|07-01-21
06-1788|H|7|06-06-23
06-1700|A|22|06-06-09
2
06-2815|J|0|06-11-07
06-3110|K|1|06-12-12
06-2412|A|42|06-09-25
07-403|L|1|07-02-05
06-3251|C|1|06-12-25
06-3066|F|0|06-12-07
06-2879|A|5|06-11-15
3
07-4114|M|3|07-04-27
07-2341|N|0|07-08-27
06-985|K|14|06-03-21
05-002951|O|38|05-12-02
05-10843|O|7|05-12-06
05-2957|O|30|05-12-05
4
05-982|J|0|05-04-01
05-3007|K|0|05-12-08
05-246|E|5|05-01-24
05-207|C|17|05-01-20
5
04-2639|O|1|04-12-26
05-368|A|2|05-02-01
05-131|Q|1|05-01-13
05-797/960|R|3|05-03-14
04-002363|K|0|04-12-02
05-464|S|10|05-02-14
05-58|O|4|05-01-06
05-14|T|7|04-12-24
04-2652|C|0|04-12-27
6
07-3366|U|2|07-12-27
07-3347|U|9|07-12-27
7
07-003356|V|1|07-12-27
07-3254|W|1|07-12-17
07-3327|Y|0|07-12-23
07-3321|G|0|07-12-22
8
05-220|E|50|05-01-20
03-360|C|1|03-02-16
03-704/825|E|7|03-03-24
03-628|F|11|03-03-14
03-470|F|0|03-02-27
9
02-2720|O|1|02-12-31

B)GII.3

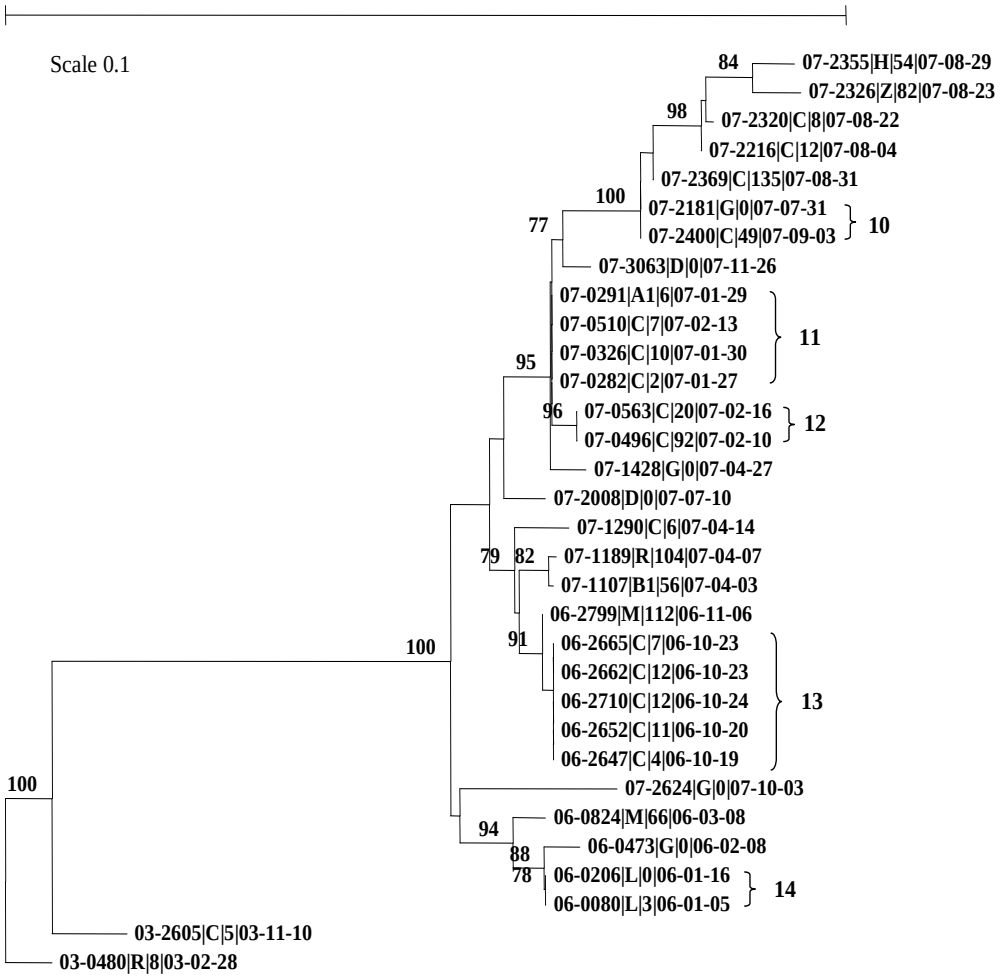


Fig 1. legend A) & B). Phylogenetic trees representing the clusters (1 to 14) of GII.4 (A) and GII.3 (B) strains from both community-acquired and nosocomial cases detected in hospitalized patients. Each strain is labeled as follows: SS-TTTT_U_V_WW-XX-YY, where SS is the year, TTTT is the unique case code, U identifies the ward, V is the number of days after admission at the time of diagnosis, and WW (year)-XX (month)-YY (day) is the sampling date. Strains from patients who were not hospitalized but were sampled while visiting the outpatient clinic or emergency department are indicated with a V of 0.

Statistical analysis

To test for differences in proportion of nosocomial infection, the following steps were taken. First, the proportion of nosocomial cases within all cases (based on the cut-off: onset of norovirus illness > 4 days after admission) was calculated for the genotype categories GII.4, GII.7, GII.3, remaining genotypes (rest), and unknown. These were calculated separately for the young children (0-5 years) and the remainder (>5 years), as young children potentially are at increased risk for norovirus infection and therefore virus introduction into a hospital is more common for this age group. In these calculations, we excluded cases who had been diagnosed between 2 and 4 days after hospitalizations, as the distinction between hospital-acquired and community acquired infection is not always possible (n=44). Secondly, using the Chi-Square test of independence, we tested whether the proportion of nosocomial infection was independent of (a) genotype, within each age group, (b) age, within each genotype, (c) genotype, within all ages and (d) age, within all ages. Because we were testing multiple hypotheses (nine in total), we needed to adjust the Chi-Square p-values to control for false discoveries. We use the Benjamini-Hochberg method here, as this method has more power than other Bonferroni-type procedures (2). A relationship was considered significant if the adjusted p-value did not exceed 0.05.

Results

In total, 264 patients (out of n = 2458 that were evaluated) had tested positive for noroviruses during the 5 year period. Of these, 61 % of the infecting strains (n=160) could be genotyped. Viruses belonging to GII.3 (34%, n = 54), and GII.4 (51%, n = 82) were most commonly identified, followed by viruses of GII.7 (9%, n=15), GII.2 (4%, n=6) and GI.6B/ II.17 (2%, n=3). The samples that could not be genotyped were retested using different diagnostic PCR's. Mean CT values did not differ between samples that could or could not be genotyped.

Overall, 48 % (n=128) of NoV positive patients most likely had hospital acquired infection according to the cut-off. Newly diagnosed cases 17 % (n=44) had onset of illness within 2-4 days after admission, but their exact source of infection could not be established. Finally, 35 % (n=92) tested NoV positive 0-1 day after admission and

were classified as community cases. In Table 1 the proportion of nosocomial cases for several groups based on age and genotype is shown. These proportions vary from zero (for GII.7 strains in adults) to 0.737 (for GII.3 strains in children below 5 years of age). As shown in table 1, genotype GII.3 in children showed a high proportion of nosocomial infections, whereas in the age group > 5 this was the case for GII.4 strains (Table 2). Testing the relationship between the proportion of nosocomial transmission and genotypes on one hand, and age on the other hand showed that the proportion of nosocomial transmission was significantly different in the older age group, but not in children. Overall, nosocomial NoV was more commonly observed in young children. Viruses of genotype II.3 were more often found in young children.

Table 1. Proportion of nosocomial infection by age and genotype[a].

Group	Genotype	Age	Proportion of Nosocomial infection	No. of Patients
1	II.7	0-5	0.286	7
2	II.7	Rest	0.000	6
3	II.4	0-5	0.478	23
4	II.4	Rest	0.553	47
5	II.3	0-5	0.737	38
6	II.3	Rest	0.143	7
7	Unknown	0-5	0.688	48
8	Unknown	Rest	0.694	36
9	Rest	0-5	0.500	6
10	Rest	Rest	0.000	2
A	All genotypes	0-5	0.631	122
B	All genotypes	Rest	0.531	98
C	II.7	All ages	0.154	13
D	II.4	All ages	0.529	70
E	II.3	All ages	0.644	45
F	Unknown	All ages	0.690	84
G	Rest	All ages	0.375	8

[a] Groups are defined as any combination of age group and genotype. Patients who tested positive for NoV after 2 to 4 days of admission were classified as indeterminate and were omitted from the analysis (n = 44).

Table 2. Results of testing the relationship between the proportion of nosocomial transmission and genotypes or age.

Within subgroup	*Null Hypothesis*	*Adjusted P-value*	*Significant*
0-5 year old	Independent of Genotype	0.14	
>5 years old	Independent of Genotype	<0.01	Yes
GII.7	Independent of Age	0.69	
GII.4	Independent of Age	0.69	
GII.3	Independent of Age	0.02	Yes
Unknown	Independent of Age	>0.99	
Rest	Independent of Age	0.60	
All ages	Independent of Genotype	<0.01	Yes
All genotypes	Independent of Age	0.30	

Molecular clustering

Based on clustering of cases in time and place (two or more cases on the same ward within a five day interval) 22 clusters had previously been identified in the original dataset (1). Viruses from the two major genotypes of NoV (GII.4 and GII.3) were further analyzed. Sequence comparison of amplified P2 domains showed nine clusters of GII.4 strains involving 17 different patients and five clusters of GII.3 strains involving 8 different patients (Figure 1 and Table 3). Of the molecular clusters, three (two GII.3 and one GII.4) had previously been identified through epidemiological observation as shown in table 3. The other 11 identified clusters of patients had not previously been identified as such. This was explained by the fact that all clusters included patients from different wards and ages. Remarkably, for 5 patients this included a link with a patient that had visited the hospital outpatient care department but was not admitted (clusters 3, 6, 9, 10 and 14)

Discussion

We describe results of a systematic evaluation of patients diagnosed with NoV in a large hospital between 2002 and 2007, to look for evidence of nosocomial outbreaks through sequence based clustering of cases. This approach was done as part of a study aimed at mapping the sources of virus introduction that may be amenable to intervention strategies, as NoV outbreaks in hospitals may have significant health impact. The use of sequence analysis in this study identified 11 clusters that had not been recognized through earlier defined epidemiological clustering (1), increasing the number of probable nosocomial clusters by 50 %. Almost half of these involved links with a patient that had visited the hospital but was not admitted, suggesting introduction of virus into wards through staff movement or contaminated surfaces. As we used a rather conservative cut-off for the definition of nosocomial infection, we may have underestimated the prevalence.

Table 3. Overview of molecular clustering versus epidemiological clustering

Molecular clusters	Presence of epidemiological clusters	Ward (s)	Presence within 5 days	No. of nosocomial infections	No. of indeterminate Cases	No. of community -acquired cases
1	NO	Different	Yes	2	0	0
2	NO	Different	NO	2	0	0
3	NO	Different	NO	1	0	1
4	YES	Same	Yes	3	0	0
5	NO	Different	Yes	2	0	0
6	NO	Different	Yes	1	0	1
7	NO	Same	Yes	1	1	0
8	NO	Different	Yes	0	0	2
9	NO	Different	NO	2	0	1
10	NO	Different	NO	1	0	1
11	NO	Different	NO	3	1	0
12	YES*	Same	Yes	2	0	0
13	YES*	Same	Yes	4	1	0
14	NO	Same	NO	0	1	1

*Cluster identified by both methods, but the size (number of patients) differed

We analysed the virus diversity in relation to date of hospitalisation. This provided the opportunity to compare diversity of the viruses in nosocomial patients with that of the viruses causing illness in the community. This comparison is essential, as widespread community outbreaks may occur in which case finding identical sequence in the hospital may not signify a hospital acquired event. An example where this occurred is cluster 8 in Fig. 1 (A), showing 2 apparently connected patients with community acquired illness. However, as all other community acquired cases were distinct, this strengthened the support for the observed approach and thus the clusters that were identified.Our findings clearly show the limitations of the commonly used epidemiological clustering, where these clusters would not be noticed. Here, patients are identified as possible linked cases when they have been hospitalised within the same wards and within the same time frame (5 days).

A limitation in our study is the number of samples for which genotype could not be determined. As mean CT's did not differ between stool samples with and without a genotyping result, quantity of virus in the original specimens is not an explanation. A reasonable explanation could be the different PCR's used for diagnosis and genotyping: the former uses a smaller amplicon size and fragmentation of RNA during preparation and freeze-thawing could preferentially influence the genotyping PCR with its longer target fragment. Alternatively, it is possible that the non-typeable samples contain different norovirus genotypes, but we could not find any evidence for that.

In the current approach, we used a stringent selection based on 100 % similarity among strains as defining a link (24, 25). This may be too stringent, as NoV is rapidly evolving, and mutations are accumulated rapidly (20). Therefore, allowing one or even two nucleotide differences between sequences could potentially increase the sensitivity of outbreak detection. However, this remains to be proven, as few studies have addressed the evolution of NoV over different chains of transmission (3, 20).

Interestingly, we found that the proportion of nosocomial infections seems to depend on the particular strain involved. In particular the GII.3 strains showed a significantly higher proportion of nosocomial infection regardless of age compared to the other genotypes. This illustrates the complexity of NoV epidemiology, showing that NoV should not be viewed as "a" virus, but rather as a group of related viruses with

different properties. This comes as no surprise given the huge diversity of NoV: viruses belonging to GII7 and GII.4 are quite distinct. Taking these differences into account, one could possibly speculate that each specific genotype could be associated with a particular disease burden. As shown within the family *Picornaviridae*, another family of positive stranded RNA viruses, genetically related viruses can cause a quite distinct spectrum of diseases (13). In addition to the virus genotype, age group needs to be factored in: the GII.3 strains are found more often in children and in nosocomial cases, compared with GII.4 for which this age difference was not found.

Our findings suggest either differences in susceptibility or severity of GII.3 in different age groups, as has been described for group A rotaviruses (16). A plausible explanation would be the development of herd immunity, given the widespread circulation of these viruses. For GII.4, rapid evolution of viruses into new antigenic variants has been shown to be an explanation for the repeated epidemics involving all age groups (14).

The age related probability of transmission in a healthcare setting is something to be aware of. The generally higher rate of nosocomial infection in the young is easily explained by the hygienic conditions: young children may wear diapers and the handling thereof is associated with higher exposure to stools. Without proper hand washing hygiene, this may constitute a greater risk of transmission. A second factor could be that viral loads are higher in young children as has been observed for other viruses in which immunity develops. Be it as it may, non-viral factors, such as behavior (e.g. hygiene) seem to be an important factor contributing to transmission.

In conclusion, we show the usefulness of molecular information as basis for detecting transmission events in the hospital setting. We show that the use of molecular typing may increase the early detection of clusters by 50 %, and were able to identify introductions from the outpatient department. This indicates that a careful review of movements of people between outpatient clinics and wards could potentially identify areas for improvement. The significantly increased proportion of nosocomial transmission of GII.4 and GII.3 strains compared with that of other NoV belonging to other genotypes shows that an early warning system that identifies increasing prevalence of new variants of these genotypes rapidly could be used to guide enhanced infection control policies.

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Nosocomial transmission of norovirus is mainly caused by symptomatic cases

Chapter 4

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Abstract

Background. Nosocomial norovirus (NoV) infection is common and may increase the burden of disease in health care settings, particularly in vulnerable hospitalized patients. Implementing effective infection control during and after admission may limit further spread, but evidence based measures are lacking.

Methods. In this study we performed a systematic evaluation of sources and modes of transmission during norovirus outbreaks within two types of healthcare facilities. An outbreak protocol was developed to sample all patients and healthcare workers (HCW) with and without symptoms on wards involved in outbreaks. Data on clinical history and possible high risk exposures were collected. Five outbreaks were investigated, involving 28 patients with recognized symptomatic norovirus infection.

Results. Enhanced sampling, however, yielded 65 additional cases, of whom 14 % (n=9) were asymptomatic patients, 57 % (n=37) symptomatic HCW, 17 % (n=11) asymptomatic HCW. For 12 % (n = 8), clinical data were not provided (2 HCW and 6 patients). On the basis of the shedding kinetics, the onset of infection was estimated for each case. The generation interval was then used to construct plausible transmission pathways and reproduction numbers for symptomatic and asymptomatic patients and HCW.

Conclusion. We found that symptomatic patients and HCW were more often involved in transmission events than asymptomatic shedders. Asymptomatic HCW rarely contributed to transmission, despite high levels of fecal virus shedding.

Key words: nosocomial transmission, symptomatic shedders

Introduction

Norovirus (NoV) is the most common cause of acute gastroenteritis worldwide [1]. Transmission of NoV most often occurs through direct contact with NoV shedders, or indirectly through environmental or food contamination with human feces or vomit, especially in closed settings such as hospitals, nursing homes, cruise ships and hotels [2-4]. In a previous study we demonstrated the high transmissibility of NoV in a large tertiary care hospital, resulting in frequent nosocomial outbreaks [5]. Transmission from chronic patients shedding NoV for a long period of time may also occur, as we have demonstrated previously [6].

There are few examples of contribution of asymptomatic shedders to (nosocomial) outbreaks [7, 8], although several publications have reported the high prevalence of asymptomatic shedding [9-12]. In adult volunteer studies, 32 % (13 of 41) of infected persons remained asymptomatic after challenge with Norwalk virus [13, 14], but this involves persons with good general health and a rare genotype, not representative for the hospitalized group of patients. Systematic studies addressing the role of asymptomatic carriage of NoV and its implications in these settings are lacking. Implementation of stringent infection control measures is recommended to control outbreaks but scientific evidence underpinning these measures is missing [15]. One of the most frequently asked questions is when an infected healthcare worker (HCW) can return to work, particularly because people may shed NoV for several days and even weeks after recovery [16, 17]. In the present study we have investigated the contribution of symptomatic and asymptomatic patients and HCWs to transmission based on data collected during in depth investigation of three NoV GII.4 outbreaks in three separate healthcare settings. The onset of shedding was estimated from shedding patterns using a nonlinear regression model, and the results were used to identify plausible transmission patterns. This analysis strongly suggests that most transmission is caused by symptomatic shedders, and that asymptomatic NoV positive healthcare workers only minimally contribute to the spread of infection in an outbreak setting. We also conclude that there is substantial underdiagnosis of NoV infection in HCW and that these undetected infections may contribute substantially to outbreaks, particularly when they are symptomatic.

Methods

We developed an enhanced outbreak investigation protocol focusing on the identification of possible sources and modes of transmission of NoV within a tertiary care hospital and 2 nursing homes in the region of Rotterdam (Netherlands) between January 2009 and March 2010. Directors or chiefs from centers reporting 2 or more cases of NoV confirmed by polymerase chain reaction (PCR) within the same ward within the same week were asked to participate in this study. The study protocol included random sampling irrespective of symptoms of all patients and HCWs on affected wards using a strict protocol as is done for methicillin-resistant *Staphylococcus aureus* [18]. Persons involved in the outbreaks who met the inclusion criteria of the study protocol were tested weekly until a negative test was returned. Measured virus concentrations (cycle threshold/ CT measurements) in the feacal samples were used to calculate the most probable first day of shedding by means of regression analysis. Details are provided in the appendix. With the onsets of shedding known for all sampled subjects, serial intervals between all pairs of infected subjects could be calculated. Subsequently, we computed the probability of transmission between any pair of cases by using the distribution of serial intervals as described previously [19]. With this transmission matrix, reproduction numbers were estimated for symptomatic and asymptomatic patients and/or HCW.

The study was done in one academic hospital, and two nursing homes covered by the municipal health service of Rotterdam-Rijnmond. An outbreak was defined as two or more cases of patients or HCW in the same department with gastroenteritis symptoms within 24 hours. Gastroenteritis was defined as diarrhea and/or vomiting (two or more episodes within 24 hours) that could not be attributed to underlying illness, or medication.

The duration of each outbreak was defined as the period starting seven days before the start of symptoms of the index case and lasting until seven days after the start of symptoms of the last case. For this period, all patients and HCW involved with the care and treatment of patients, or using a shared toilet in the treatment facility or healthcare unit were approached for participation. Patients who had been discharged within the seven days before the onset of illness in the first recognized case were not

approached. Children up to 18 years of age and participants with a legal representative could be included in the study after authorization of their parents and/or legal representative.

Each person who consented to participation in this study was provided with a stool sampling kit and a small questionnaire aimed to consider their clinical state. Quantitative reverse transcription-PCR and genotyping of the strains were performed as previously described [5]. Persons with NoV positive stool specimens were asked to resend a specimen once a week until they tested negative.

Data analysis

Estimation of onset of excretion in asymptomatic cases

In order to calculate likely transmission patterns, an estimate of the onset of infectiousness is needed for each case. In previous studies using only symptomatic cases, this has been assumed to be the date of illness onset [19], but this approach can not be used for the asymptomatic shedders. Therefore, we estimated the first day of shedding from the time course (increase followed by decrease) of virus excretion using stool sample data that were available for both asymptomatic and symptomatic cases. A parametric model of virus shedding was used, based on a compartment model of virus production and excretion in the intestinal tract ("Appendix"). After transformation to –log scale (to accommodate for CT measurements as obtained by real time PCR analysis) the model was fitted in a two-level Bayesian framework, allowing for variation in time course (rates of increase and decrease) and amplitude (peak level of virus excretion) among individual shedders. Onset of shedding, measured as time from the first sample, was included as a covariable that could be estimated for both symptomatic and asymptomatic shedders. More details of the model and parameter estimation are available in the "Appendix".

Transmission Patterns

With the estimated onsets of shedding, asymptomatic cases could be added to the dataset, extending the epidemic curve. With the help of the serial interval distribution (defined as the distribution of time between onsets of shedding in successive symptomatic or asymptomatic cases) the onset dates may then be used to estimate the likelihood of transmission between any pairs of cases, as reported previously [20]. Different outbreaks were treated as separate (isolated) clusters, and no transmission was assumed to occur between cases infected with different genotypes. In contrast to patients, the HCW cannot be assumed to be in continuous contact with hospitalized patients or nursing home residents. To adjust for contact time, the transmission probabilities of HCW were weighted by the fraction of the time they were present (typically 8 of 24 hours). No difference in prior weighting was applied for symptomatic/asymptomatic cases. Pairwise probabilities of transmission (the elements of the transmission matrix) were estimated by Markov chain Monte Carlo sampling, as reported previously [19], resulting in a Monte Carlo sample of matrices with elements representing the probabilities of transmission from any subject (*i*) to any other subject (*j*).

Different iterations of this matrix were used to obtain uncertainty estimates for any of the selected output metrics. Initial parameter values for the serial interval distribution were taken from a previous study [19]: a gamma distribution with parameters (shape parameter 3.35, scale parameter 1.09, leading to a mean serial interval of 3.6 days, and mode (most likely interval) 2.6 days). As soon as convergence was reached, adjustment of the serial interval distribution was allowed by updating its parameters, followed by optimization of the transmission matrix until (again) convergence. This nested approach resulted in improved estimates (higher posterior probability) with slightly shorter estimates of the serial interval compared with its initial value.

Transmission by category

The number of infections caused by any subject *i* was estimated by summing over all cases *j* the probability that *j* was infected by *i* [20]. This is defined as the reproduction

number of subject *i*. Thus transmission among and between patients and HCWs, and from patients to HCWs and the reverse, were calculated as reproduction numbers of these categories for each of the outbreaks. We further stratified the analysis by type of symptoms, differentiating between cases with diarrhea and cases with vomiting

Results

Outbreak analysis

From January 2009 until March 2010, five outbreaks were reported caused by three different NoV genotypes i.e. GII.4, GII.2 and GII.7. One cluster (OB 1) was segregated by use of molecular typing, into 2 groups of patients infected with GII.4 2008 (n= 18) and GII.4 2006b (n = 4), respectively (Table 1). Because the GII.b strain sequences were diverse, these patients were excluded from the group as likely new introductions unrelated to the outbreak. Full details of this outbreak will be published elsewhere. All the other outbreaks consisted of a single genotype. The duration of the outbreaks ranged from 38 days to 77 days. Outbreaks (OB) 1, 2 and 3 were detected in the hospital, whereas 4 and 5 occurred in nursing homes. In total 386 HCW and 127 patients/residents were approached for participation, and 257 (66 %) of the HCW (range 60-72 %) and 79 (77 %) of the patients/residents (range 68-100 %) agreed to take part in the study. In total, 50 (20 %) of participating HCW had at least one positive stool (PCR), and 43 (54 %) of participating patients were tested positive. Details on testing results are listed in table 1. The enhanced case finding resulted in a 276 % (n=69) increase in total number of subjects with confirmed NoV infection, assuming that typically only symptomatic NoV in patients/ residents are reported, as has been detected in the routine diagnostics. The shedders that were symptomatic detected in each OB were as follows: OB 1 (n=16), OB 2 (n=6), OB 3 (n=8), OB 4 (n=18) and OB 5 (n=17). Of the additionally diagnosed shedders, 29 % (n=20) did not report any symptom.

Table 1. Details of Norovirus outbreak investigations, with number of persons recruited and included, as well as outcomes of stool testing for health care workers (HCW) and patients or residents (P) involved in the outbreaks.

				Number						
	Healthcare center	**Duration of outbreak**	**Genotype**	**Approached (HCW/ P)**	**Compliant (HCW/ P)**	**PCR pos (HCW/ P)**	**Symptomatic (HCW/ P)**	**Asymptomatic (HCW/ P)**	**Unknown clinical status (HCW/ P)**	***PCR neg (HCW/ P) with symptoms***
OB1	**Tertiary care hosp.**	**31 Jan / 10 Mar '09**	**GII.4- 2008/ GII.4-2006b[a]**	**64/ 21**	**46/ 16**	**15/ 7**	**11/ 5**	**4/ 0**	**0/ 2**	***4/ 0***
OB2	**Tertiary care hosp.**	**25 Mar/ 04 Jun '09**	**GII.2[a]**	**69/ 22**	**48/ 15**	**0/ 9**	**0/ 6**	**0/ 2**	**0/ 1**	***9/ 2***
OB 3	**Tertiary care Hosp.**	**14 Oct/ 09 Dec '09**	**GII.7[a]**	**107/ 5**	**64/ 5**	**9/ 1**	**7/ 1**	**0/ 0**	**2/ 0**	***7/ 1***
OB4	**Nursing home**	**10 Jan/ 25 Feb'10**	**GII.4- 2010**	**88/ 26**	**57/ 20**	**14/ 10**	**11/ 7**	**3/ 1**	**0/ 2**	***8/ 9***
OB5	**Nursing home**	**5 Feb/ 23 Apr '10**	**GII.4-2010**	**58/ 28**	**42/ 23**	**12/ 16**	**8/ 9**	**4/ 6**	**0/ 1**	***6/ 2***
Total				**386/ 102**	**257/ 79**	**50/ 43**	**37/ 28**	**11/ 9**	**2/ 6**	**34/ 14**

Abbreviations: HCW, health care workers; OB, outbreaks; Neg, negative; P, patient or resident; PCR, polymerase chain reaction; Pos, positive

[a]These genotypes (variants) are not included in the modeling

Modeling

The analyses described below were done only for the GII.4 outbreaks (OBs 1, 4 and 5), because transmission patterns may differ for viruses belonging to different genotypes, thus precluding pooling of all data [5, 6]. GII.4 strains are predominant in outbreaks in healthcare settings [21]. The same serial interval distribution was used for all outbreaks, because there is no information at this point about the heterogeneity in serial intervals among NoV GII.4 variants. The shedding kinetics of all the cases involved in the GII.4 outbreaks was used to estimate onsets of shedding. Transmission analysis produced adjusted estimates of the serial interval distribution, and estimates of the probability of transmission between any pairs of cases in the studied outbreaks. An example of a transmission tree derived from the transmission matrix iteration with the highest posterior probability (as approximation of the posterior mode) is shown in Figure 1.

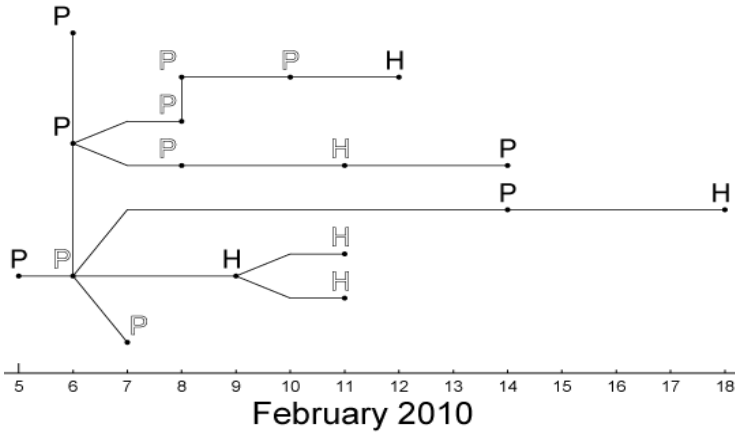

Figure 1: Example of a plausible transmission tree for one of the outbreaks (OB5), based on outcome of markov chain monte carlo as described in the "Methods" section. Patients/residents (P) and healthcare workers (H) are shown, symptomatic cases are indicated in black characters, and the asymptomatic cases are shown as gray characters

The Monte Carlo estimates for the probability of transmissions by any infected subject were used to calculate their individual reproduction numbers and these could be averaged by category, stratifying cases by symptom status for NoV infected persons for all cases without diarrhea (white boxes; Figure 2 left panel) this resulted in an average reproduction number of 0.85 (95% confidence interval 0.55-1.05). And for all cases with diarrhea (grey boxes; Figure 2, left panel) this resulted in an average reproduction number of 1.64 (95% confidence interval 1.56-1.70). This shows that the contribution of asymptomatic shedders to transmission was significantly lower than that of symptomatic individuals when data from all outbreaks were combined, although the pattern differed between outbreaks: in outbreak 4, the difference in contribution to transmission from asymptomatic cases was not significantly lower. Next, we stratified the data for patients and HCW (Figure 2, right panel). For the symptomatic patients this resulted in an average reproduction number of 1.89 (95% confidence interval 1.71-2.12). And for the symptomatic HCW this resulted in an average reproduction number of 1.30 (95% confidence interval 1.08-1.52). While for the asymptomatic HCW no reproduction numbers could be detected. This result shows that the difference was greatest for the HCW, where contribution to spread by asymptomatic persons could not be detected, and for symptomatic HCW this was significantly lower than for symptomatic patients. Asymptomatic patients did contribute to transmission, but less than persons with symptomatic infection.

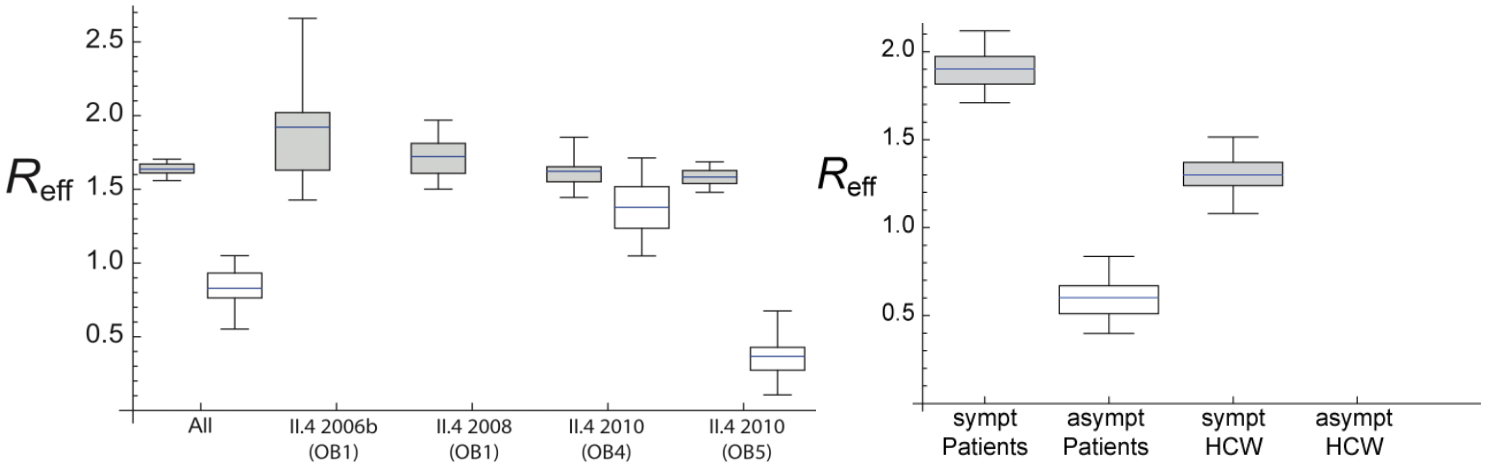

Figure 2. The box plots indicate (from the center outward): median, quartiles (25 and 75 percentiles) and 95% interval (whiskers, 2.5 and 97.5 percentiles). Left: estimated reproduction numbers for each outbreak for cases with (gray) and without (white) symptoms (diarrhea). Right: estimated reproduction numbers for all outbreaks, stratified for symptomatic (gray)/asymptomatic (white) staff (healthcare workers [HCW]) and patients, with and without symptoms (only the first 5 days of all outbreaks). In outbreak 1, transmission from asymptomatic cases did not occur and no transmission was found from asymptomatic HCW, therefore these boxes are missing. Abbreviation: Reff, reproduction numbers.

Conclusions and discussion

In this study, we performed structured outbreak investigations with enhanced sampling following after notification of a suspected outbreak, to obtain data that can be used to design evidence based strategies for prevention of spread of NoV in healthcare settings. We have seen that enhanced sampling yielded a 232 % increase of identified shedders consisting of asymptomatic patients and HCW, and symptomatic HCW that normally would be missed in the routine sampling. These results illustrate the potential underestimation of the size and duration of an outbreak in regular outbreak investigations where sampling of HCW with health complaints is not done routinely, and sampling to identify asymptomatic shedders is rare.

HCW in general have been presumed to play a role in transmission, because their work involves direct contact with many patients. We used the enhanced surveillance data to address the question of who contributes most to shedding. Persons infected with NoV may continue to shed high amounts of virus for several weeks after resolution of symptoms [5, 6, 22], and hospital hygienists or persons working in the food industry need to decide whether it is safe to return to work. The current Center for Infectious Disease guideline recommends that people should not resume work until 2-3 days after clinical recovery, but evidence for this is lacking and the extra leave of absence may be problematic in small food establishments or health care settings during peak seasons. Therefore, we tried to determine who contributes most to the spread of infection, and whether this was related to being symptomatic.

Our findings clearly suggest that symptomatic shedders are more frequently involved in transmission than asymptomatic shedders. No transmission was observed from asymptomatic HCW, despite considerable levels of virus shedding in the stool. This shows that an infected person does not need to be infectious, most likely related to proper personal hygiene. The latter is indicated by the difference between HCW and patients with NoV: there was a significant difference in the contribution of HCW to transmission both for symptomatic and asymptomatic shedders, and the awareness of HCW of the need for hygiene would be the most likely explanation for this difference. For instance, proper hand hygiene can lower the incidence of NoV [23], although overall evidence for effects of hand hygiene on prevention of healthcare associated infections is considered to be weak [24]. Nevertheless, our observations suggest that a

more important factor is the health status. Our study did not reveal differences between persons with vomiting and persons with diarrhea in their contribution to spread of infection, as is frequently suggested. This may be related to the power of the study, and should ideally have been addressed in more extensive studies. However, these studies are difficult to conduct, because the occurrence of outbreaks cannot be planned, and willingness to participate in studies is influenced by the extra work needed in patient care during outbreaks. Based on these findings, we conclude that the period of mandatory leave from work for infected HCW potentially may be relaxed. However, this conclusion relies on unbiased reporting of illness episodes by HCW, which may be overly optimistic. In a study of self compliance during a NoV outbreak, it was found that a quarter of affected personnel did not adhere to quarantine recommendations [25].

Illness reports from hospitalized patients and from HCW are often done through different reporting channels (for instance the hospital hygiene department and the office of personnel), although the combination of observations would enhance the early detection of outbreaks. Our study shows that notified cases in an outbreak may be the proverbial tip of the iceberg, and increased awareness is warranted.

Even with enhanced sampling there is still evidence of under ascertainment: of the HCW that did agree to participate, 13% of those that tested negative for NoV did have symptoms compatible with acute NoV infection. There are several possible explanations why no virus was found. Sampling of these cases may have been too late, so that virus excretion may have decreased to undetectable levels [16]. In addition, HCWs may be capable of early clearance of the virus due to their better immunity status compared with hospitalized patients [26]. Therefore, we compared the results with and without the NoV negative HCW with diarrhea. Adding them to the transmission analysis produced similar results for the distributions of reproduction numbers in different subject categories (as in Figure 2, results not shown).

An important factor that was not included in our study and may influence the construction of transmission trees is the role of environmental contamination. This is a potential source of protracted outbreaks, as has been documented in multiple outbreak reports [27-30]. In our approach, we disregarded this option, by assuming relatively short intervals between consecutive cases, based on distribution of generation intervals observed during person to person outbreaks. In the outbreaks studied here,

we believe that environmental persistence did not play a major role, because the observed generation intervals plotted based on the dates of onset of new cases did not differ significantly from the distribution observed during a model outbreak [19]. Of course, this cannot be taken as a generalization, because situations may differ between hospitals and healthcare settings, resulting in vast differences in the likelihood of environmental contamination [27, 30, 31].

To our knowledge, this is the first time estimates of the first day shedding of asymptomatic cases with NoV have been made. Virus excretion patterns were only used for determining the onset of shedding in symptomatic and asymptomatic shedders. As a further refinement it is tempting to use other aspects of the excretion patterns, such as duration of shedding and the peak levels of excreted virus, as proxies for the infectivity of individual cases. In a subsequent study we plan to enhance the transmission analysis method to incorporate these additional information sources, allowing for individual differences in infectivity to improve the identification of likely transmission pairs.

To conclude, effective guidelines are needed for diagnostics that includes HCW and contacts of positive cases (enhanced screening). Awareness of reporting symptoms by the HCW and patients is still an important element for rapid outbreak detection to possibly achieve timely intervention.

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Potential conflicts of interest.

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Chapter 5

Shedding of norovirus in symptomatic and asymptomatic infections.

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Abstract

Background and methods. Enhanced surveillance for studies of transmission of norovirus in hospital outbreaks has yielded a considerable number of asymptomatic shedders. Fecal samples from symptomatic and asymptomatic infections were analyzed by real–time PCR. To study norovirus shedding a quantitative dynamic model was fitted to the shedding data from 102 subjects, in a multilevel Bayesian framework.

Results. The results indicate that shedding in asymptomatic cases is similar to that in symptomatic infections, both showing considerable variation in peak levels (average 10^5 – 10^9 per g feces) as well as the duration of virus shedding (average 8 – 60 days). Patients appear to shed higher numbers of virus than staff, for slightly longer durations, but the differences are too small to be significant.

Conslusion. The results are of interest for studies of norovirus transmission, specifically studies that need to quantify the deposition of virus in the environment.

Keywords: norovirus, shedding, asymptomatic infection, viral gastroenteritis, quantitative PCR

Introduction

Viral gastro–enteritis is an important concern for public health and a major cost factor for society. Hospitals and nursing homes are affected in particular [1, 2]. The epidemic potential of norovirus is striking [3, 4] and has been partly attributed to its high infectivity [5]. There is no clinical treatment for norovirus disease, or a vaccine that protects against infection, although clinical trials are ongoing [6]. Hygiene measures and quarantining of cases are therefore currently the only option for containing an outbreak [1, 4]. Norovirus is transmitted via the fecal–oral route: primary food– or waterborne cases may shed virus into their environment. Virus deposited on surfaces can then cause outbreaks [7, 8] and virus can be transferred by hand contact [9] followed by ingestion [10]. In order to better understand how much virus is deposited in the environment, we have studied virus shedding in infected subjects.

A human challenge study has shown that there may be considerable heterogeneity in the numbers of viruses that are shed by infected subjects [11]. Generalization of the results from this study to nosocomial outbreaks raises uncertainty because of study size (16 subjects) and the use of experimental infections, with a Norwalk virus (GI.I) inoculum in healthy volunteers [12].

For the present study into shedding of norovirus during nosocomial transmission, patients with symptoms of acute norovirus infection as well as any known contacts were asked to submit fecal samples that were analyzed by quantitative RT–PCR for the presence of norovirus [13, 14].

Materials and methods

Data

Four distinct outbreaks of gastro–enteritis caused by norovirus GII.4 were detected by prospective monitoring during the winters of 2009–2011, in a tertiary care hospital and three nursing homes [14]. Upon detection of outbreaks of two or more PCR confirmed cases in the same ward, data were collected from as many patients and

health care workers as possible, with or without symptoms of norovirus gastroenteritis [14]. Fecal samples were collected from all subjects who gave consent and these were followed up weekly, until they tested negative for norovirus.

Virus concentrations were measured by quantitative (real time) RT–PCR. Details about sample collection and preparation, and assay performance have been published [15]. For any sample a single Ct value was always measured.

Four NoV GII.4 outbreaks studied in the winters of 2009 – 2011 were selected for further analysis, yielding a total of 230 fecal samples in 102 (out of a total of 125) subjects. Of these, 47 were patients (32 with symptoms, 15 asymptomatic) and 55 health care workers (39 with symptoms, 16 asymptomatic). There were also 23 subjects (6 patients and 17 health care workers) with symptoms of gastroenteritis who did not consent to collection of samples.

Model for the time course of virus shedding

A realistic model of the time course of virus shedding must include an initial increase in virus concentration, followed by a decrease to undetectable levels. For virus produced at an infection site and transported by bowel movement, the observed virus concentration in feces can be written as

$$C(t|\alpha,\beta) = C_0 \mathrm{e}^{-\alpha t}(1-\mathrm{e}^{-(\beta-\alpha)t}) \quad (\beta > \alpha) \tag{1}$$

At onset of shedding (t = 0) the virus concentration $C(t)$ increases from 0 (with rate $C_0(\beta - \alpha)$), reaches a maximum and decreases to zero (with rate α). More details, as well as a tentative biological mechanism, are given in the online appendix, section A.

Assuming the parameters (C_0,α,β) vary among individual infected subjects, this model can be applied in a Bayesian hierarchical framework [16].This results in individual estimates of the time course of virus shedding, with the variation among these individuals characterized by (joint) probability distributions of the parameters of the regression model (equation 1). All models were run in JAGS (v 3.3.0) [17] using rjags (v 3-10) [18] in R (v 3.0.1) [19]. Due to censoring tests of model fit using the DIC

cannot be used, but in a (separately provided) supplemental file the reader can check that the model accurately fits the observed Ct data. Figure A1b in the appendix shows residuals for all observed Ct values in all subjects.

The onset of shedding (t = 0 in equation 1) cannot be observed. For symptomatic subjects the onset of symptoms may be assumed to occur shortly after the onset of shedding [14]. For asymptomatic subjects an estimate of the onset of shedding may be found that provides the best agreement of the observed virus titres with the time course of equation 1. This has been achieved by expressing time of onset of shedding relative to the first fecal sample that was collected, and treating the delay between onset and first sample as a parameter that can be estimated. Technical details of this procedure are given in the online appendix, section B. For the symptomatic subjects the estimates of shedding onset agreed well with the observed symptom onsets, as shown in the online appendix of [14].

Calibration curve

To obtain a titration curve a standard suspension was made of RNA prepared as run–off transcript from cloned fragments (GII.4 Bristol region B, junction ORF I and II). To avoid inhibition on the target RNA, yeast tRNA was added that competitively can react with the RNase. For quality control a dilution series in yeast tRNA was included until 10^{-15}. The number of genome copies in an undiluted PCR sample of that suspension can be calculated from the RNA concentration measured by Nano Drop (ND–1000 ISOGEN Life Science) as follows: an equivalent volume of 0.33 μl (2/15 × 2.5μl) of the RNA solution contains 2279 ng/μl RNA, with 1684 nucleotides (MW ≈ 1684×320 +159 =539.04 kD). The concentration of genome copies in undiluted suspension then is

$$2279 \times \frac{10^{-9}}{10^{-6}} \times \frac{0.33 \times 10^{-6}}{539.04 \times 10^{3}} \times N_{\mathrm{Av}} \approx 8.487 \times 10^{11} \text{genome copies}$$

where N_{Av} is Avogadro's number (6.02214×10^{23}). Tenfold serial dilutions of this standard suspension were used for obtaining a calibration series of Ct values.

A calibration curve, obtained by linear regression, allows translation of observed Ct values in fecal samples into numbers of genome copies of NoV (GII.4). In the regression procedure, Ct values of 40 were considered censored (Ct ≥ 40). Figure A1c shows the regression curve with (95%) prediction intervals. A Monte Carlo sample of the estimated parameters of this regression model was used to translate Ct estimates into virus concentrations, incorporating measurement error due to translation.

Results

Fecal samples containing norovirus were obtained from 71 symptomatic and 31 asymptomatic subjects. Numbers of patients/staff and their age ranges are given in Table 1.

	Total	Symptomatic		Asymptomatic	
	number	number	age (mean, 95% range)	number	age (mean, 95% range)
patients	54	32	81.6 (50.6 – 93.6)	15	80.4 (55.8 – 94.2)
staff	47	38	40.7 (18.2 – 61.3)	16	35.0 (18.9 – 53.2)

Table I: Numbers of infected patients and staff sampled for norovirus shedding, by symptoms, and their ages (mean age in years and 95% range).

Using the date of the first fecal sample as a reference, estimates of the period Δt_{pred} were obtained: the time between onset of shedding and the first sample. In symptomatic cases with fecal samples present there is good agreement between Δt_{pred} and observed times between the onset of symptoms and the first sample, indicating that symptoms tend to appear within a day after the start of virus shedding.

In asymptomatic subjects the onset of symptoms is missing, and the fitting procedure thus produces estimates of the onset of shedding, based on observed virus shedding patterns [14].

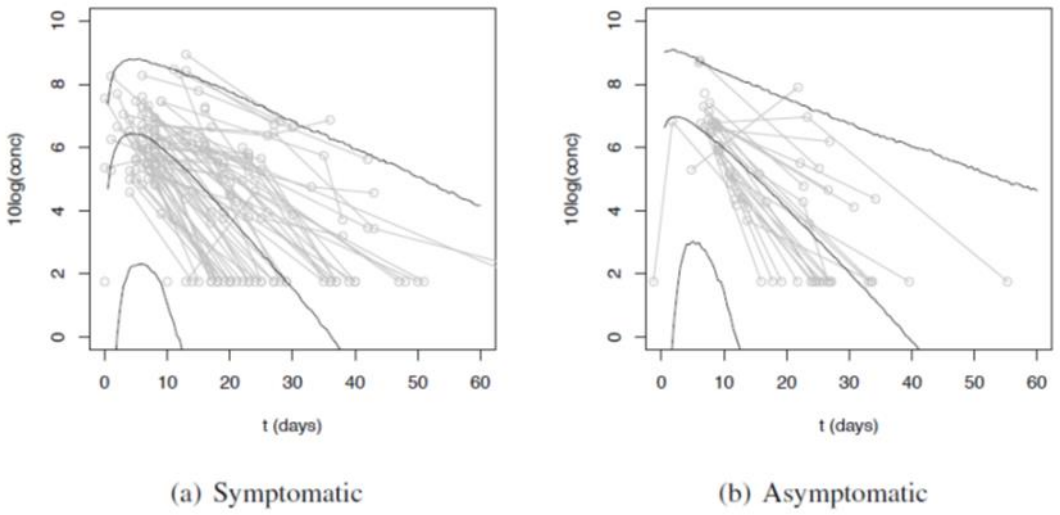

Figure 1: Time course of virus shedding in symptomatic and asymptomatic subjects. Median and 95% interval of the predicted virus concentration (10 log numbers of viruses per g stool) are shown

Figure 1 shows predicted levels of virus shedding as a function of time, in symptomatic and asymptomatic subjects. More details are given in the online appendix ; fitted responses for all individuals can be provided in a separate document.

For ease of interpretation, instead of rates of increase and decrease the time from onset of shedding to peak levels and the duration of shedding (time from onset of shedding to decline to Ct = 40, considered the diagnostic detection limit) can be calculated (Figure 2b,c). In addition, the peak levels of virus shed (Figure 2a) and the total amount of virus (area under the shedding curve) can be calculated (Figure 2d).

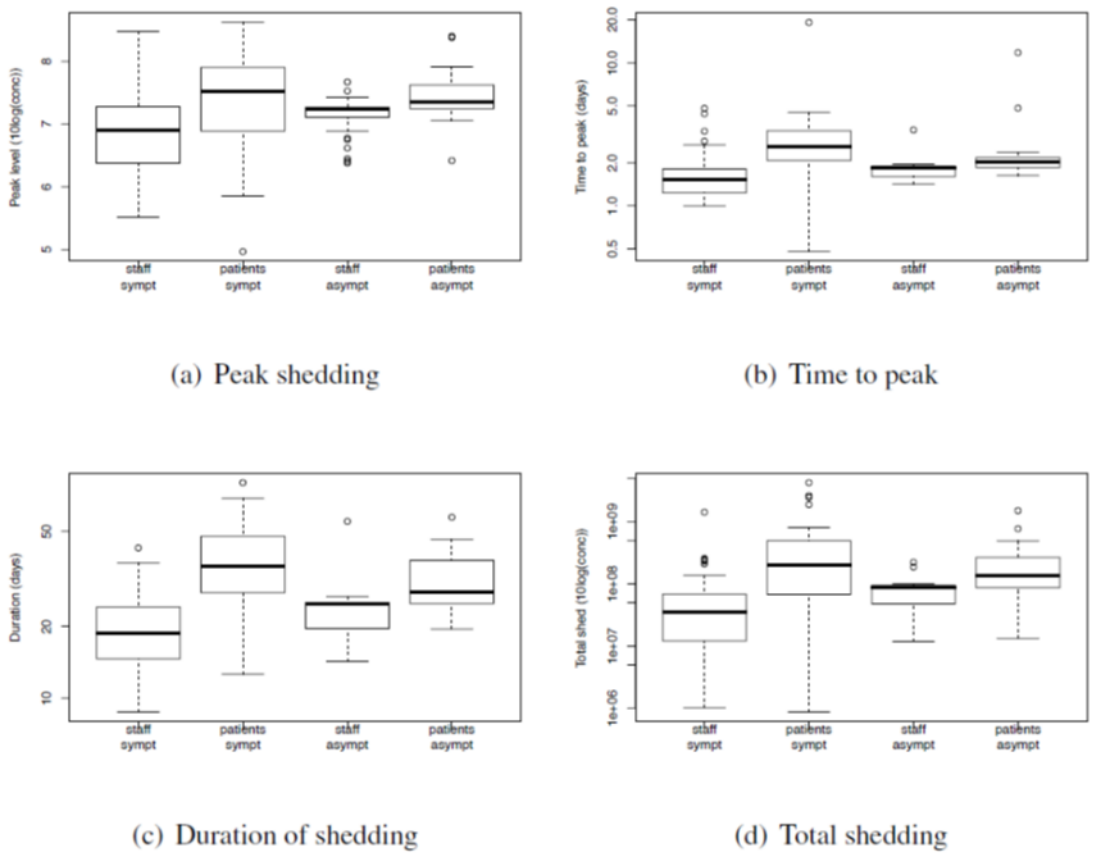

Figure 2: Characteristics of virus shedding for patients and staff: estimated means of (a) peak levels of shedded virus (10 log number of viruses/g stool), (b) time from onset of shedding to peak in virus shedding (days), (c) total duration of the shedding period (days) and (d) the estimated total amount of virus shed (area under the shedding curve, 10 log numbers of viruses/g stool). Means of Monte Carlo samples of (posterior) values, for staff and patients, symptomatic and asymptomatic.

There is an indication that patients, in particular when they have symptoms, may shed higher levels of norovirus, for somewhat longer periods (Figure 2). Note however that Figure 2 shows the variation in individual means of predicted characteristics: when uncertainty is taken into account the differences are very small (see p–levels in Table A2 in the appendix).

The posterior distributions of these characteristics also do not indicate a substantial difference between symptomatic and asymptomatic subjects. There is however considerable uncertainty, as illustrated in the individual estimates in Figure 3.

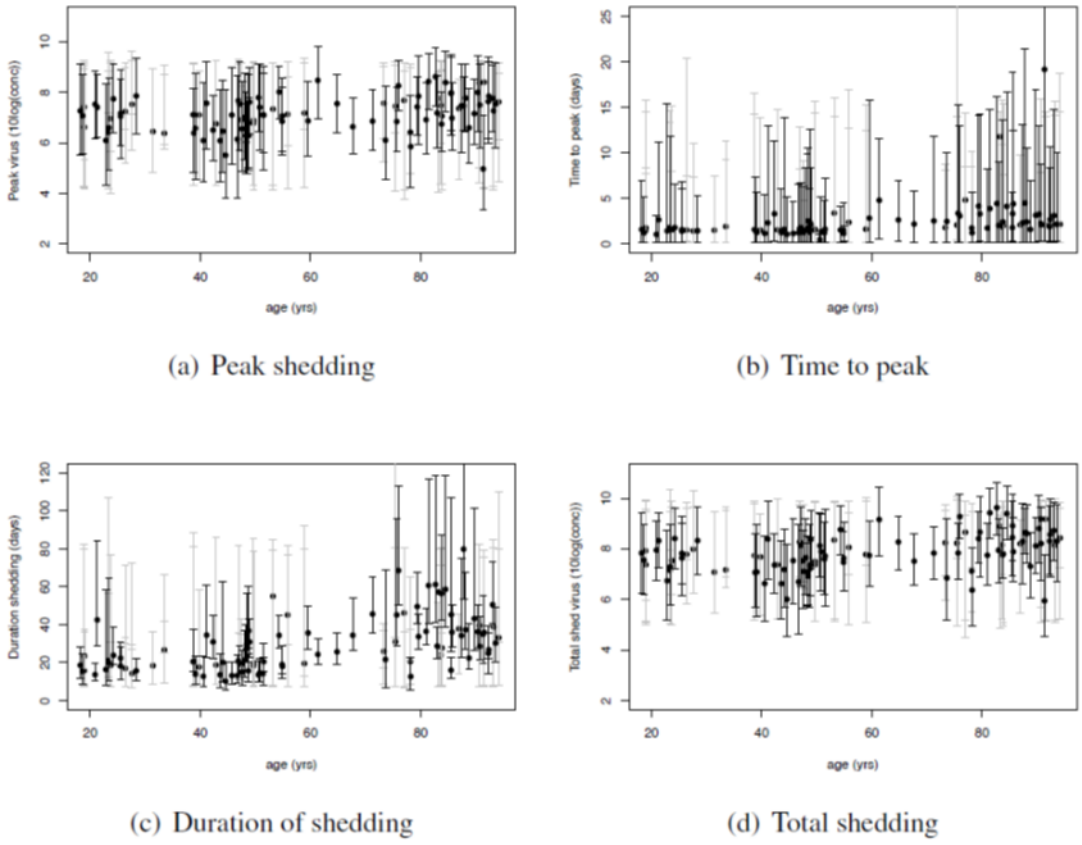

Figure 3: Characteristics of virus shedding vs. age of individuals: estimated individual (a) peak levels of shedded virus (10 log numbers of viruses/g stool), (b) time from onset of shedding to peak in virus shedding (days), (c) total duration of the shedding period (days) and (d) the estimated total amount of virus shed (area under the shedding curve 10 log numbers of viruses/g stool). Monte Carlo sample of (posterior) values with mean and uncertainty (95% credible intervals), for symptomatic (black) and asymptomatic (grey) subjects.

None of the four characteristics of the time course of shedding (peak levels, time to peak, duration of shedding and total excreted virus) appears to depend on age of the infected subjects (Figure 3). Estimated time to peak and durations of shedding show positive correlation (Pearson correlation coefficient 0.873 and 0.973 for symptomatic and asymptomatic subjects, respectively), as do peak levels and total amount shed (correlation coefficient 0.960). For additional details see the appendix (Table A1).

Discussion

Norovirus is transmitted fecal–orally: its high infectivity and persistence in the environment allow the virus to exploit various pathways. Norovirus present on fomites is considered to be important for transmission [10]. Such virus can be deposited by infected subjects, via contaminated hands [9]. As a potential driver for the numbers of viruses that are deposited by infected subjects, the concentration of virus shed in feces is of interest. A study of virus shed by volunteers challenged with Norwalk virus (GI.1) showed considerable variation in the numbers of viruses in stools of infected subjects [11]. Another study of nosocomial norovirus infection in aged–care facilities showed a range of concentrations and durations of shedding [20].

The present study comprises a larger number of subjects than previous studies, including some with asymptomatic infections, sampled in the course of a study on nosocomial transmission of norovirus in the Netherlands [14]. Patterns and quantities of shedding in infected symptomatic and asymptomatic subjects are remarkably similar. The variation in peak levels among individual subjects is large, with minimum as low as few hundreds of virus genomes per g, and maximum (95%–ile predicted concentration) above 10^{10} virus genomes per g feces. The peak excretion levels are in the same range as those reported by [20], and slightly lower than those reported by [11]. It remains unknown whether the difference may be attributed to the different genotype (GI.I) used in the latter study.

The rates of increase and, more importantly, decrease of virus shedding are also variable, but to a lesser extent. In infected subjects shedding has dropped to low levels (less than 10^3 viruses/g) by day 60 post infection. As symptoms usually do not last longer than 2 weeks the contribution of such long lasting shedding to transmission may remain small [14].

In a study among hospitalized patients more serious symptoms (longer duration) were associated with higher virus concentrations [21]. In the study reported here such an association was not apparent, in fact, symptomatic subjects seem to shed norovirus in similar numbers as asymptomatic subjects (Figures 3a and 3d), while both categories show considerable variation (mean peak levels of excreted virus/g stool vary over a range of $\approx 4_{10}$ log units).

One may be tempted to interpret the shedded numbers of viruses as an indicator for the intensity of infection, with higher numbers corresponding to more infected cells in the intestinal mucosa. If that is true, the variation in numbers shed may not have great clinical significance because shedding appears equally intense in symptomatic and asymptomatic infections. Staff, either symptomatic or asymptomatic, seems to shed higher levels of norovirus than patients, for a longer period. However, the difference is small and insignificant against the large variability in responses (Table A2 in the appendix).

Also worthy of note is that a transmission study in the same cohort has shown that symptomatic cases (staff and even more so patients) are more likely to transmit the virus than asymptomatic shedders [14]. As asymptomatic shedders appear to produce equal numbers of viruses as symptomatic cases, this difference in transmission must be attributed to enhanced environmental seeding of the virus, associated with vomiting and diarrhea.

It is likely that only a part of the excreted virus genomes is in infectious virus particles, as has been observed for other viruses [22–24]. Assuming that the fraction infectious virus does not depend on the level of shedding (the peak concentration), this large variation may imply that different infected subjects may shed very different numbers of infectious noroviruses into their environment, and consequently they may have considerably different infectiousness to their contacts.

The present study is part of a greater project aimed at quantifying nosocomial transmission of norovirus in hospital and care facilities. It will be interesting to use the individual shedding patterns as a proxy for the infectiousness of each subject, allowing adjustment of the contribution of any individual to transmission according to the numbers of viruses they excrete at any time following infection [4, 14, 25]. Such an individual–based approach may considerably improve the faithfulness of tracking of infections during an outbreak [26]. Direct use of the estimated virus concentrations to predict risk of transmission from individual infectious subjects seems attractive. It must however be realized that at present little is known quantitatively about the role of human behavior both in spreading shedded virus in the environment and in picking up that virus from contaminated fomites.

Conclusions

During norovirus infection, high numbers of virus are shed in feces. Numbers of viruses in feces increase rapidly, reaching peak levels within a few days following onset of infection, followed by a slow decline for several weeks. It may take more than two months until fecal concentrations of virus reach undetectable levels. Both peak levels and duration of shedding show considerable individual variation that is not different between symptomatic and asymptomatic infections.

Conflicts of interest and funding

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The authors declare that, to our best knowledge, there are no conflicts of interest, financial or otherwise, that may have influenced the work described here. A preliminary version of the results reported here has been presented at the Fifth International Calicivirus Meeting in Chile, 2010.

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P2 domain profiles and shedding dynamics in prospectively monitored norovirus outbreaks

Chapter 6

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Abstract

Background. Norovirus P2 domain is commonly used to extrapolate transmission within an outbreak (OB) setting. The current definition is that transmission among cases is considered to be proven when no sequence variation is found.

Objectives. Previous studies have shown a high mutation rate and errors during replication of the norovirus genome, therefore the validity of this criterion must be evaluated.

Study design. Sequences of the P2 domain were obtained from patients and health care workers sampled during 4 prospectively GII.4 outbreaks. Faecal samples were tested by RT-PCR for presence of norovirus RNA against a standard control preparation to allow quantification. Estimated time of onset of shedding was derived from shedding kinetics modelled on data from sequential sampling. Thereby P2 sequence variation could be linked to estimated total virus excretion in individual subjects.

Results. In all the outbreaks, P2 domain variation was found that resulted in unique codon changes in some patients. Mutations were found in 14% of initial samples and > 50% of follow-up samples taken from patients involved in an outbreak. In three patients, aa mutations was observed in or near sites involved in host or antigen binding.

Conclusions. We concluded that P2 domain variation increases with duration of virus shedding, but was unrelated to total amounts of virus shed. Therefore, we propose that cluster identification based on identical sequences should be relaxed to accommodate minor sequence variation. When using sequence data to support outbreak investigations, sequence diversity should be interpreted in relation to timing of sampling since onset of illness.

Keywords: norovirus transmission, shedding, P2 domain

Background

Noroviruses (NoV) are a major cause of gastroenteritis worldwide and are most commonly associated with outbreaks in health care settings [1]. Onward transmission of noroviruses is common when guidelines for outbreak control are not applied rigorously [2]. For developing effective control measures, a proper understanding of the transmission patterns during outbreaks is needed, including the role of healthcare workers and asymptomatic shedders. Molecular typing of NoV-positive stool samples can be used to determine links between individual cases. A systematic analysis of genome diversity in a large dataset collected through the Food-borne viruses of Europe network concluded that the optimal target for sequence-based linking of cases was the capsid gene [3]. For practical reasons, currently the P2 domain of the NoV is used [4, 5, 6, 7]. This genome region is considered to be the most variable part of the genome since it codes for the protruding domain of the capsid protein, which contains the receptor binding domain and important epitopes targeted by antibodies that inhibit binding [8, 9]. In GII.4 NoV, the P2 domain evolves by accumulation of mutations under selective pressure from host immunity [10, 11, 12]. Accumulation of mutations in this domain was also shown in immunocompromised patients with prolonged shedding of viruses [13]. Recently the use of next generation sequencing identified minority variants present during transmission events [14].

Objectives

P2 domain sequencing has been used for identifying the transmission pathways and links during outbreaks [4-7] and identical P2 domain sequences are considered evidence for a cluster. However, with the high mutation rate of norovirus [15], nucleotide changes may occur within a short time interval, raising the question what would be an appropriate minimum number of nucleotide changes for defining a cluster of cases connected by direct transmission links. This question is relevant because outbreaks may be missed with common cluster detection algorithms that use time and space, or pseudo-outbreaks may occur when many patients are hospitalized during peak season [6, 7]. Therefore, we set out to quantify P2 domain variation, during four prospectively monitored outbreaks in three nursing homes and a tertiary care hospital [16]. We

sampled NoV-positive patients and health care workers (HCW), identified through an enhanced outbreak protocol irrespective of their symptom status. Variation in sequence data between and within outbreaks, as well as variation between and within infected subjects was analyzed and correlated with the estimated number of viruses shed by each individual. The results can be used to redefine criteria for linking of cases to outbreaks.

Study design

We prospectively monitored four GII.4 outbreaks starting from January 2009 until March 2011 in the region of Rotterdam in the University Hospital (EMC) and in nursing homes [16]. Sampling was based on an enhanced outbreak investigation protocol focusing on the identification of possible sources and modes of transmission of NoV [16]. The study protocol included random sampling irrespective of symptoms of all patients and HCW on affected wards with NoV. Patients and their contacts involved in the outbreaks who met the inclusion criteria of the study protocol were tested weekly until a negative test was returned. Each case was confirmed by sequencing region A (genotyping) followed by P2 domain sequencing [6, 17]. The amount of virus shed by each subject was estimated from real time RT-PCR analysis of all fecal samples, using RNA transcribed from a plasmid containing a sequence spanning all commonly used diagnostic targets as a reference template [18], allowing us to investigate correlation between virus excretion and P2 domain changes over time. Background sequences from the same geographic region were collected from patients outside the studied outbreaks, with NoV infection detected 2 days after admission.

Sequence analysis

RNA fragments were reverse transcribed with random hexamers (Invitrogen), yielding cDNA that was amplified by a nested PCR and subsequently sequenced using the ABI-PrismBigDye Terminator v3.0 Ready Reaction Cycle kit. The same primers were used for amplification and sequencing the P2 domain (primers 1st PCR: F: 5'gangatgtcttcacagtctctt '3, R: 5'cattcctgggggagtagaca '3 [4], Nested primers: F: 5' gtgccacccacagttgag '3, R: 5'gggagtagacagtccaa '3). DNA Sequences were entered and

assembled in bionumerics 6.6.2 (Applied Maths, Sint-Martens-Latem, Belgium) and evaluated manually for their quality by looking for the number of ambiguities, errors, mismatches and deletions.

Sequences were aligned; genotype and variant assignment was based on the RdRp region [16] using the norovirus typing tool (http://www.rivm.nl/mpf/norovirus/typingtool; [19]. Full-length P2 domain sequences (600 nucleotide) were then subjected to pairwise analysis (UPGMA) to identify strains linked to the same outbreak, and by advanced cluster analysis (maximum parsimony), to compare diversity within and between outbreaks and robustness of clustering. Sequence diversity within patient and between patients within an outbreak was assessed by comparing the minimum and maximum number of mismatches for each outbreak separately. Translated sequences were reviewed to look for possible directional amino acid mutations.

Sampling and virus shedding

To study the effect of sampling delay, the time of onset of shedding was estimated by extrapolation from modelled shedding kinetics, based on data from all subjects with follow up samples (Teunis et al, submitted). An RNA standard template was used to translate CT values in fecal samples into an estimated viral load. PCR based estimates of NoV shedding were then used to calculate total numbers of viruses shed by sampled subjects, allowing analysis of sequence variation against viral load, clinical symptoms (symptomatic or asymptomatic), and occupational status (HCW/patients). To characterize the rate of sequence variation all data were pooled and the survivor function for sequence change was calculated, using a Kaplan-Meijer estimator [20] describing the probability of any nucleotide changes versus time from onset of shedding .

Results

Strain typing and clustering

The four GII.4 outbreaks occurred in 3 nursing homes and 1 university hospital. The ages of the included HCW/ patients from the hospital setting ranged from 25-77 and 54-77 years, respectively. In the nursing homes, ages for the residents were high (72-95 years), while for the HCW this ranged from 20-63 years. Details of the outbreaks have been described elsewhere (15). In total 175 HCW and 77 patients consented to enhanced case finding, of which 50 HCW and 47 patients tested positive for NoV infection (Table 1). Capsid gene sequencing was successful in 109 NoV positive stool samples from a total of 252 sampled cases, comprising 44 HCW and 37 patients [16]. OB 4 yielded 48 sampled cases but the data is not published yet. Failed sequences were repeatedly tested but persistently failed to produce sequence information. The success of sequencing was unrelated to the levels of virus shedding (data not shown).
Phylogenetic analysis of all P2 domain sequences showed a clear discrimination of the four GII.4 outbreak clusters, but with mixed results for OB 1: here, the outbreak strains segregated into three different clusters: GII.4 2008 (17 cases) and 2 clusters belonging to the GII.4 2006b variant lineage (2 cases each), (Figure 1). As this suggests that a minority of the patients was from a different, unrelated cluster, detailed molecular analysis was not performed for these strains. Data retrieved from the hospital database showed that one of the samples belongs to a nurse who was involved in patient interviews. The other three subjects were patients who had been admitted into the hospital. From the epidemiological data, it was clear that one patient developed diarrhea after admission, indicating a nosocomial infection.

Comparison of these results against the strain diversity observed in the background dataset (defined as sequences from patients admitted with norovirus infection) showed that these were unique and distinct from the outbreak sequences with few exceptions (4 %), (Figure 1). In OB 4 a unique single case was observed who showed at least 15 nucleotide differences compared to the outbreak strain, suggestive for an unrelated introduction.

Table 1. Summary of cases and sequence data

	Location	NoV cases (HCW/P)	Nr cases with P2 domain sequence (HCW/ P)	Nr samples with Follow-up P2 domain sequences, HCW/ P	Maximum nr nucleotide changes within (Patient/ OB)*
OB 1 (GII.4 2008)	Tertiary care Hosp.	15/ 7	13/ 2	3/ 2	0/ 22
OB 2 (GII.4 2010)	Nursing home	14/ 10	15/ 8	3/ 13	2/ 2
OB 3 (GII.4 2010)	Nursing home	12/ 16	11/ 15	0/ 4	1/ 2
OB 4 (GII.4 2010)	Nursing home	9/ 14	5/ 12	0/ 3	1/ 15
Total		50/ 47	44/ 37	6/ 22	

* Including all persons initially considered being part of the outbreak

Within and between patient sequence diversity

The percentage nucleotide diversity based on P2 domain analysis (600 base pairs) between outbreaks (OBs) including all samples ranged from 6.3- 7.3 % of sequences different at genotype level, and from 0.7- 1.2 % at variant level. Sequence variation within outbreaks was small (0 – 0.3%) and overlapped with sequence diversity within subjects (0- 0.3 %). At the time of first sampling 70 (86 %) persons of OB 1-4 (both patients and HCW) had an identical sequence, designated the outbreak strain consensus, whereas 6 (38 %) were shedding a virus with a single nucleotide change in the first sample and one person had a sequence with 2 nucleotide changes shortly after onset of shedding. During follow-up, more nucleotide changes were seen; in total 56 % of follow-up samples tested yielded one or more mutations.

Figure 2 shows the timing of sampling in relation to the estimated period of shedding, as estimated from the kinetics of shedding as described elsewhere (Teunis et al., submitted). The sampling delay ranged from 0-23 days for symptomatic cases with a median of 8.5 days since the estimated time of infection, and 3-7 days with a median of 5.4 days for asymptomatic cases.

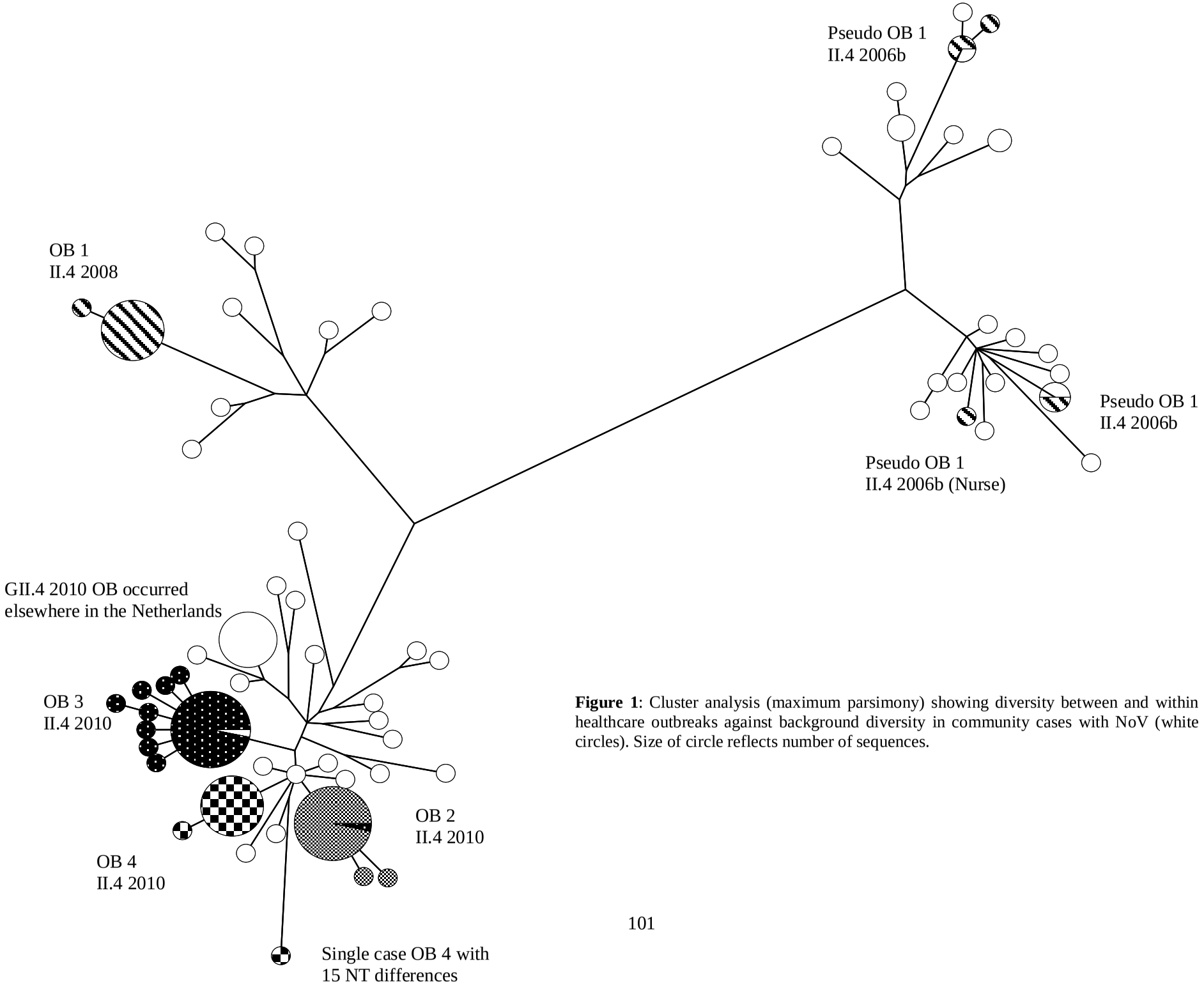
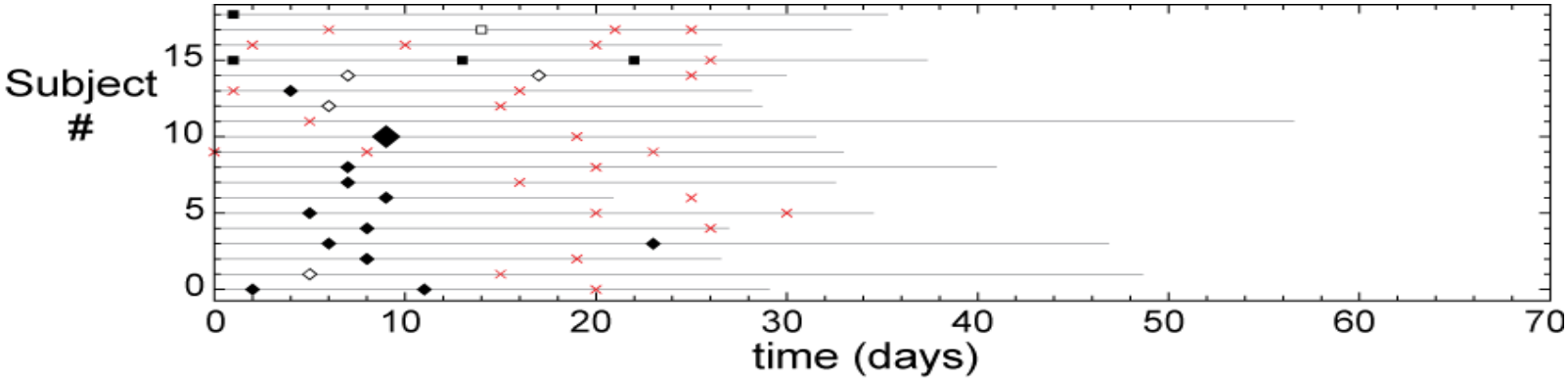


Figure 1: Cluster analysis (maximum parsimony) showing diversity between and within healthcare outbreaks against background diversity in community cases with NoV (white circles). Size of circle reflects number of sequences.

OB 1


Subject
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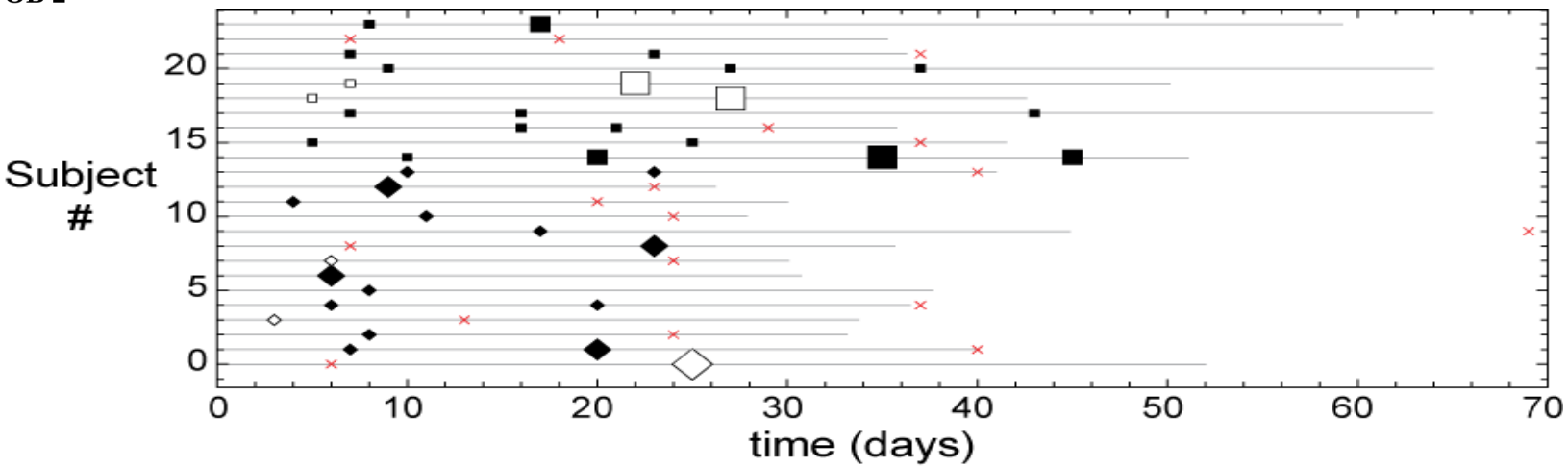
time (days)

OB 2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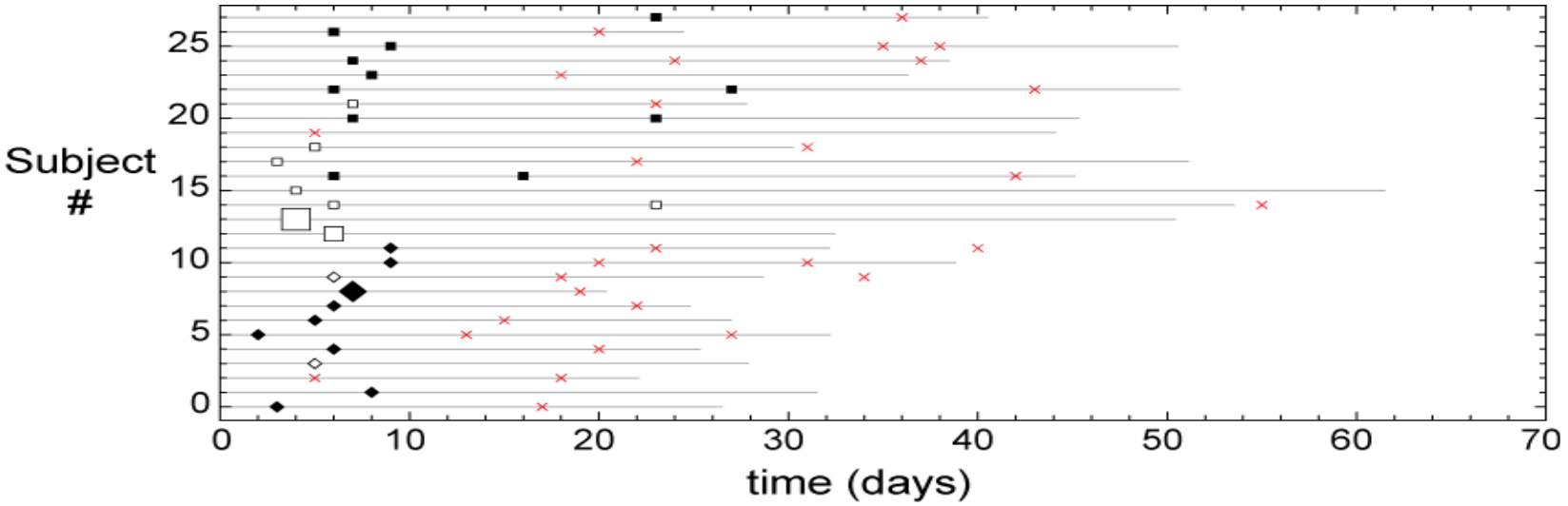
OB 3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OB 4

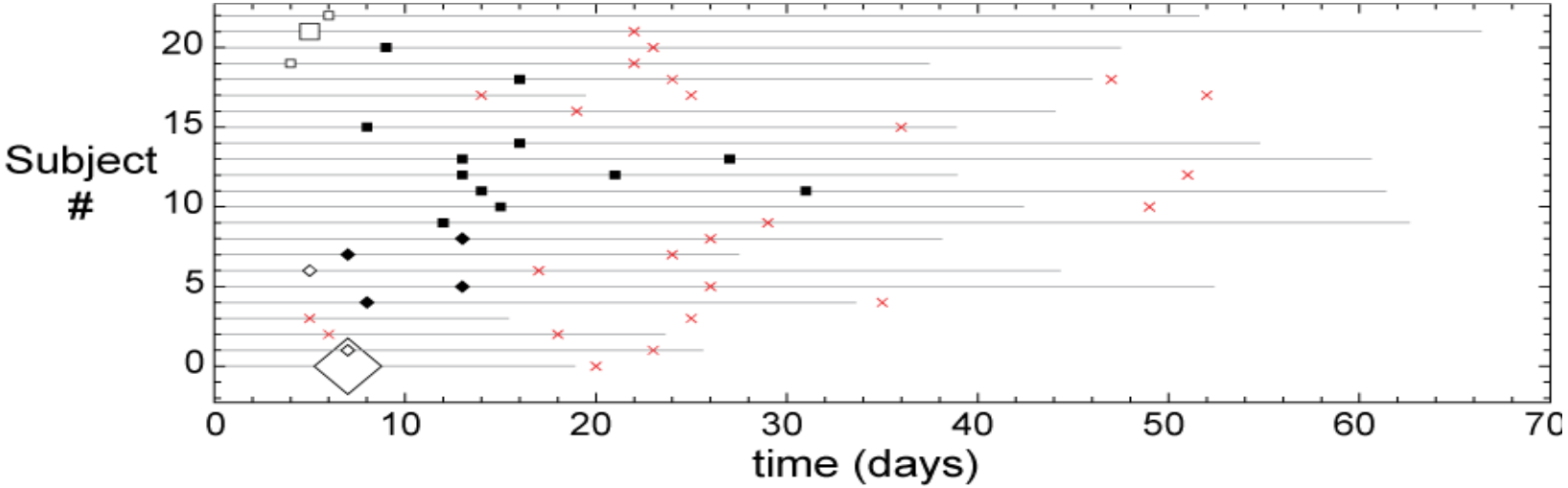
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Figure 2: Time course of virus shedding (modeled) and sequence changes for individual subjects in 4 NoV GII4 outbreaks. Squares indicate patients, diamonds health care workers; filled symbols indicate symptomatic cases, empty symbols asymptomatic subjects. Size of the symbols indicates sequence change: smallest 0 bp change, medium size 1 bp change, large symbols 2 bp change. One subject in OB 4 (bottom) has a sequence with 15 bp different (extra-large square) from the consensus sequence for that outbreak. X indicates when a sample was taken that was either negative, or for which a sequence could not be established. Horizontal lines (grey) indicate (estimated) duration of shedding, from estimated onset (t=0) to the time when the CT increases above 40

When summarizing the rate of sequence change in a Kaplan Meier plot, sequence changes appeared from 4 days post onset of shedding. The rate of increase in probability of a sequence change indicates that for samples collected at three weeks post onset of shedding there is a 10% probability of (1-2) nucleotide changes (Figure 3).

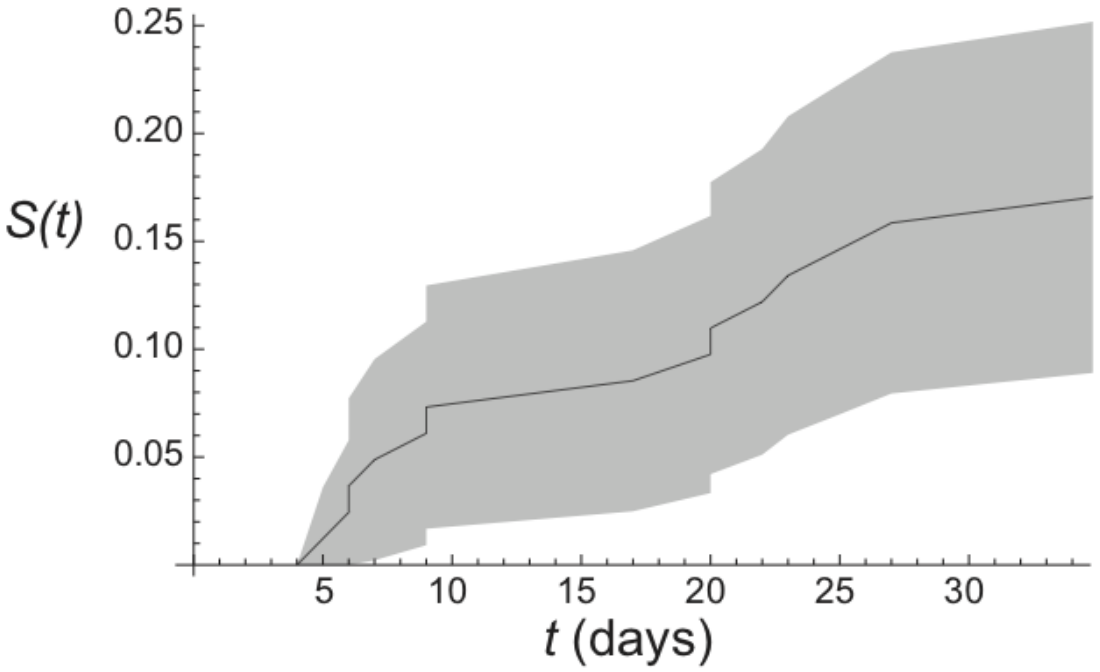

Figure 3: Sequence changes Y-as (S/ 100 %) over time after onset of shedding X-as (days), shown by nonparametric (Kaplan-Meier) estimate of the probability of sequence change calculated for the complete set of infected subjects from all four outbreaks (mean curve and 95% range).

When the shedding data were combined with the sequence data no significant association could be seen between shedding and virus excretion or sequence variation, although patients seemed to excrete slightly higher numbers of viruses than HCWs, for a longer period (Figure 4).

Codon changes

In total, 11 nucleotide changes resulted in codon changes (6 in HCW, 5 in patients). Codon changes were observed in OB's 2 and 3 only. In these outbreaks, almost all nucleotide sequence changes (8 out of 10 and 3 out of 3, respectively) were codon changes. Six of the amino acid changes were located at positions in the P2 domain that have been identified as informative sites, because they were one of the marker mutations for global variants of GII.4 (S255G, S310R, T340A, Y352H, S393N, K248R) [11, 12, 21, 22]. Amino acid changes at positions A256T and N373S were seen sporadically in the past, as illustrated in figure 5 (Genbank accession numbers). The remaining amino acid changes were unique, located at the following sites; D312N, D312E, R411K. One of the mutations was located in the histo-bloodgroup antigen-binding site (position 393), one in a position that was resulted in an additional RGD motif on the GII4 2002 strains, and one mutation was near epitope A. No pattern of amino acid changes was observed to verify transmission between subjects (Figure 5).

Previously reported outbreaks

Background information from the hospital and the nursing homes with OB 1, OB 2, OB 3 and OB 4 indicated that no outbreaks had been reported on the same department shortly before these outbreaks were identified.

Discussion

Molecular analysis can help define transmission pathways during outbreaks, particularly when combined with metadata [5, 6, 17]. For NoV the proposed molecular marker is the P2 domain, since this domain is considered hypervariable thus providing sufficient resolution for use of sequence data for linking of cases (17). According to our results, each outbreak has a unique consensus sequence based on P2 domain homology. Phylogenetic analysis of P2 domain sequence data can therefore unravel pseudo-outbreaks and specifically can serve to exclude strains that do not belong to the outbreak (Figure 1). It can also provide information regarding the extent of the outbreak, for instance concurrent sampling from the population extraneous of the outbreak can provide additional linked cases based on the P2 domain homology.
More detailed analysis of the domain revealed that in addition to diversity of the P2 domain between outbreaks there was minor variation within each outbreak, and among follow-up samples from individual patients and HCW. Most of the samples were collected between 4 and7 days of post onset illness when the transmission was most intense; however, the majority of the NT changes occurred in a later stage (Fig 2). In the outbreaks studied here, the variation within the P2 domain does not exceed 2 nucleotide changes and therefore a maximum number of nucleotide changes of 0.33 %/ 600 bp is a conservative threshold to suspect a new introduction. The time interval to the first sequence change can be relevant for future outbreak investigations, with changes observed as early as 4 days following infection.

The position of each nucleotide change was unique and was only found in single cases with one exception. This suggests that minor sequence changes during NoV outbreaks are a random phenomenon in otherwise healthy individuals. However, an indication of immune driven selection is the finding that the majority of mutations in follow-up samples were codon changes, with two remarkable mutations: mutations in position 393 have been associated with alterations in histo-bloodgroup antigen binding patterns of GII.4 strains [23], and therefore such strains potentially could target a different segment of the population. One mutation was at a position adjacent to epitope A (position 373) and therefore potentially influencing antibody binding. Amino acid changes at position 340 have been found in almost all GII4 variant transitions, and therefore may be significant as well [12, 21, 22]. While speculative, this

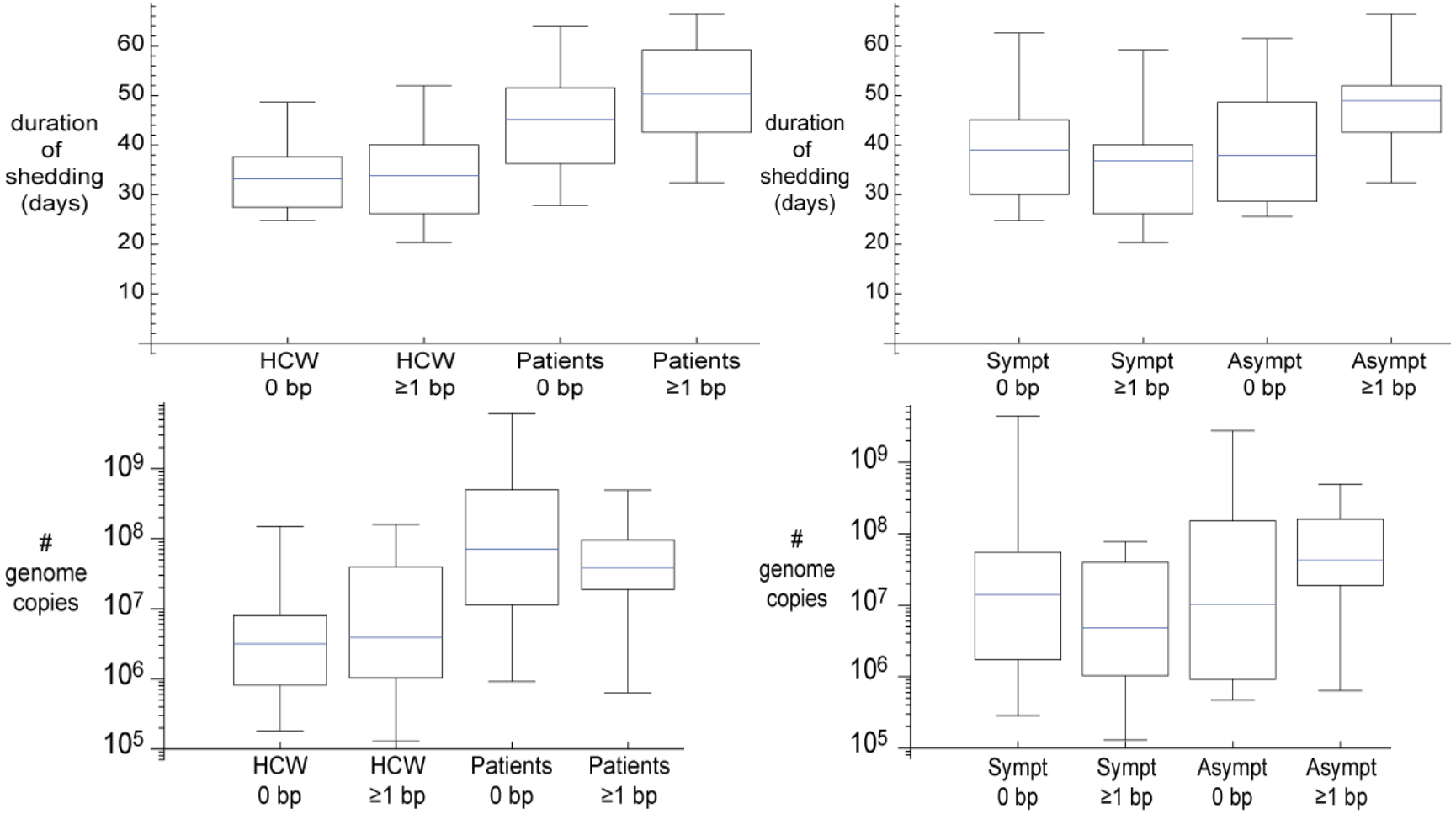

Figure 4: Box plots (arithmetic mean, quartile box and 95% whiskers) of the duration of shedding for HCW and patients without and with sequence changes, and for symptomatic and asymptomatic cases without and with sequence changes. Also shown (bottom row): same for the estimated total numbers of viruses shed.

suggests that new variants may arise quite rapidly in patients during the course of a single infection. Still, an antibody response has to be mounted after an infection since we do not know the exposure history of the individuals involved. Although all of them were adults and therefore most likely had experienced multiple norovirus infections prior to the present one, given the high incidence of these infections in the population [24].

Obviously, more information is needed to define transition to a new (epidemic) variant. In our study, we found no evidence of onward transmission of the viruses with potentially informative mutations. Without changes, affecting transmissibility such variants would most likely not emerge as major strains, given the omnipresence of competing strains circulating in the general population. This may be different when such infections occur outside the norovirus winter season, and a question is whether chronic norovirus infection in immune-compromised individuals could serve as a reservoir for new variants [13].

Finally, the sequence variations observed in the present study demonstrate the need to reconsider the guidelines [4-7] for identifying clusters: the currently used cut-off of 100 % identical P2 domain sequence should be relaxed to allow minor variations, thereby potentially increasing the attribution of cases in health care settings. Conversely, since the variation in the P2 domain is limited within the same cluster, it is often difficult to trace transmission events using only sequence data, particularly for defining transmission between individual infected subjects. We have established that the resolution is insufficient to conclusively identify links between individuals within outbreaks (who infected whom): for such purposes, more enhanced sequencing or sequencing of a larger part of the genome is required by considering the presence of minority variants/ quasi species.

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Competing interests: None declared

Ethical approval: Not required

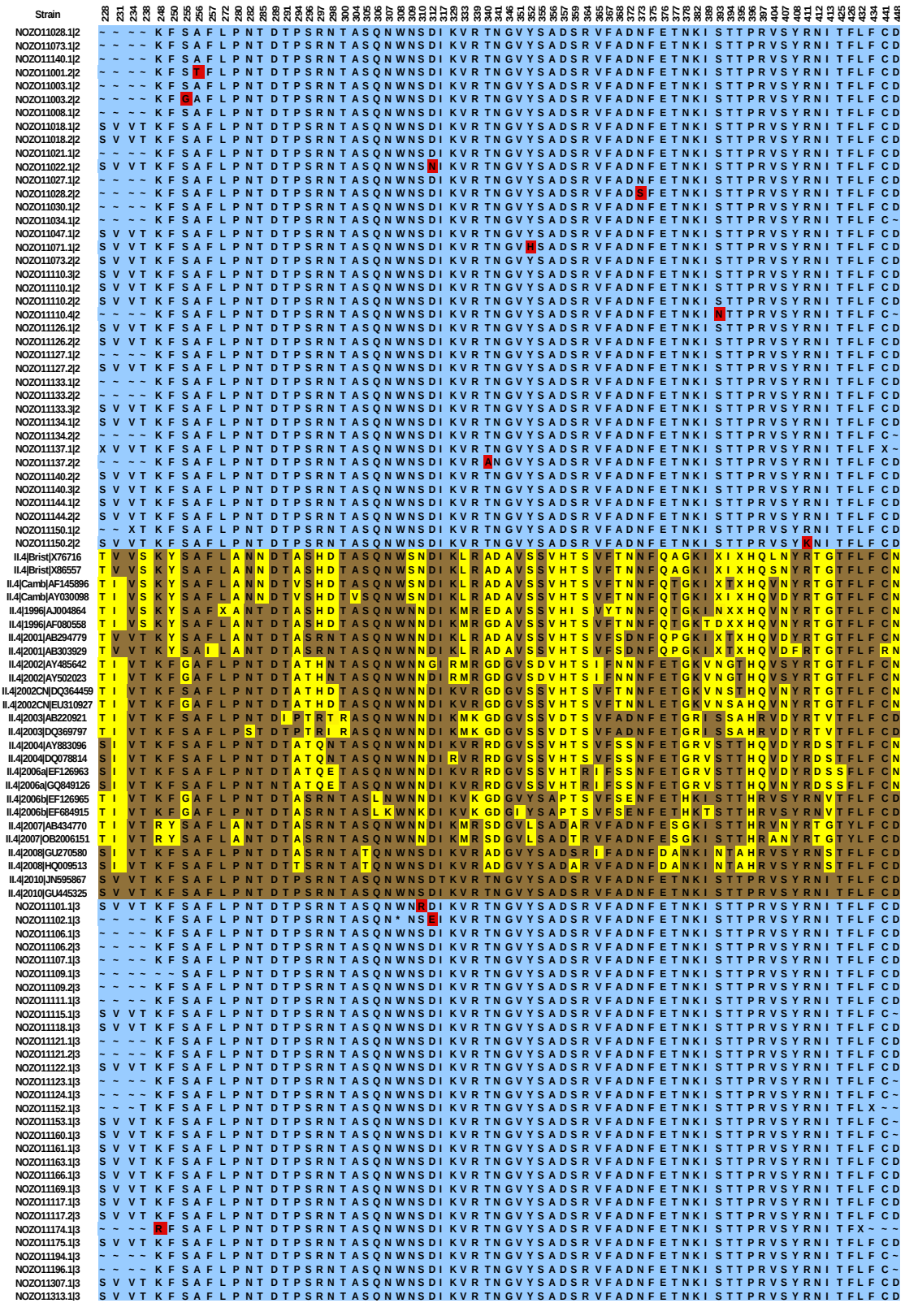

Figure 5. Amino acid (AA) changes (informative sites) in P2 domain sequences of GGII.4 outbreak strains collected between 2009 and 2010. The informative sites throughout the protein are listed from left to right. AA numbering is indicated at the top, and names are given on the left. From top to bottom, blue color indicates identical amino acids, and overlapping AA of the background (Genbank accession numbers) are illustrated in brown and distinct AA with yellow. The red colors indicate the locations of insertions of the GGII.4 strains during the OB's. Sequences in the middle area (brown) are reference GII4 strains Symbol (-) indicates failure of partial sequence.

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Sources of Norovirus Outbreaks

Chapter 7

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Abstract

Background. Noroviruses cause acute gastro-enteritis with a high rate of secondary transmission, resulting in outbreaks particularly in healthcare settings and elderly homes. Understanding sources of introduction of these viruses may provide clues for control. Here, we set out to find possible sources of introduction by combining questionnaire information with analysis of transmission patterns through laboratory and clinical reports.

Methods. From 2009 until 2011, 7 norovirus OB were investigated through an enhanced outbreak investigation protocol, where questionnaires were administered for each infected subject identified through random sampling of symptomatic and asymptomatic cases. The most likely index cases were identified by calculating transmission trees combining data on onset of illness, virus shedding, and from spatial information. The possible source of infection was assessed through review of questionnaires for these cases.

Results. The outbreaks differed in size (19-43 cases) and duration (1 – 3 weeks). In total, 199 persons consented to participate in the questionnaire survey (99 patients, 104 HCW). 60% of persons reporting with some symptoms tested positive for norovirus, with rates of positivity higher for samples collected on day's 5-8 post illness onset than earlier during the illness. In 4 outbreaks, the index case was a HCW, but 3 of these mentioned contact with a symptomatic person at work, suggesting missed cases. One outbreak most likely was patient indexed, and two outbreaks remained unresolved due to lack of information.

Conclusions. Despite extensive investigation, it proved difficult to identify sources of introduction of norovirus in the majority of outbreaks, and index cases most likely were missed in three of the outbreaks. The lower rate of PCR positivity of cases with recent onset of illness potentially contributes to underestimating extent of spread.

Introduction

Norovirus is a highly contagious virus, causing outbreaks of gastroenteritis worldwide in all age groups. Norovirus infection can be quite disruptive, causing considerable disease burden, especially in infants and the elderly [1]. Transmission of norovirus is common and outbreaks are often seen to occur in crowded environments like cruise ships, hospitals, nursing homes [2-8]. Usually the sources of outbreaks remain unclear [9]. In recent years, guidelines have been developed to target community acquired and nosocomial transmission of norovirus in health care settings, focusing on reducing onward spread [7]. Despite existing regulations and applications of different protocols to reduce transmission, there is still a need to identify sources of outbreaks [7, 10, 11]. How and when infectious subjects introduce the virus often remains unclear due to lack of detailed molecular and epidemiological information. In outbreak studies, questionnaires have been useful for collecting demographics, understanding of possible sources, behavior of subjects, introduction of the virus, and details pertaining to transmission patterns [12, 13]. Here, we present results from enhanced outbreak investigation for 7 outbreaks in the region of Rotterdam. Enhanced case finding was done by sampling of all persons involved (possibly in contact with a symptomatic case and those who consented to participate), and repeated testing of all NoV positive cases [8, 14]. A simultaneously deployed questionnaire survey was developed to complement the previously published information for 4 outbreaks, to query additional information about possible modes of transmission of NoV in these settings and to provide a standardized case history that systematically assesses possible sources (patients, personnel, visitors, environment, and food).

Materials and Methods

Set up of the study

During 2009 until 2011, 7 outbreaks have been monitored prospectively, 4 in nursing homes and 3 in the Erasmus Medical Centre, all in the region of Rotterdam (the Netherlands). A systematic sampling protocol was designed, in which patients and HCW were requested to fill out a questionnaire and to provide a weekly stool

specimen starting within one week of first notification of a case. On a weekly basis, anyone who had a positive stool sample was approached with an additional short questionnaire focusing on contact with symptomatic cases, in an attempt to verify (confirm or exclude) potential onward transmission of the virus. This protocol was continued until a negative sample was returned. Cases that tested negative and had no symptoms were not followed up and excluded from the study.

Positive samples were characterized by analysis of the capsid gene sequence (P2 domain). Sequences were aligned with the past 4 global epidemic, and with sequences from all norovirus positive cases diagnosed in the past 11 years in the same hospital to confirm or rule out clustering [7]. The main questionnaire included a standardized case history, systematically assessing potential sources (patients, HCWs, visitors, environment, food), and probable transmission routes (person to person, contaminated food, contact with vomitus/ faeces) while additional questionnaires for individual cases queried basic information e.g. age, onset date of illness and also addressed illness in (household) contacts who were asked to provide a stool specimen. Upon inclusion, each patient or HCW in this study was assigned a unique anonymized identifier code. NoV outbreaks that were notified when they involved patients (through the hospital infection prevention department) or illness reports from staff (occupational health) were treated uniformly, and one designated team member (a research nurse) contacted the hospital ward to explain the set-up of the study and interview patients and staff. The study protocol was subjected to the medical ethical committee of Erasmus MC, and was approved.

Assessment of the questionnaires

Everyone who consented to participate in the study was approached to complete the questionnaire, however less than 50 % of the questionnaires were returned (in some cases only partially completed). Details on illness onset or estimated timing of onset of shedding were used to identify most likely index cases and their contacts [15]. Review of the questionnaires from these individuals focused on possible transmission routes and potential sources of infection. Any recorded information of interventions aimed at interrupting or reducing transmission, e.g. disinfection, cleaning and quarantine, was collected, and the timing of these interventions was compared to the

epidemic curve for each outbreak. A qualitative assessment of knowledge and attitudes of HCW and patients towards hygiene measures was done through face-to-face interviews by the research nurse.

Quantitative virus detection and genotyping

To evaluate whether cases with symptoms of gastroenteritis that tested negative for NoV resulted from sampling too late or too early after onset of symptoms, all the outbreak data were pooled and the sampling delay (from illness onset) was calculated. Transmission of norovirus during each outbreak was characterized as described previously, constructing transmission networks based on serial intervals between cases [8], using estimated shedding patterns of each case (Teunis et al. submitted). These transmission networks were confirmed by similarity in molecular sequence of the viruses shed by these cases [14]. Transmission analyses allow estimation of the reproduction number for any individual: this is the total number of secondary cases caused by that individual subject.

Results

General overview and the duration of the outbreaks

Seven outbreaks were investigated. In total, 61-100% of patients / residents agreed to participation in the investigation, and 43-63% of HCW. The epidemic curves for each outbreak are shown in figure 1, listing the symptomatic and asymptomatic NoV positive cases. In addition, we also plotted persons with symptoms consistent with the case definition who tested negative (Figure 1 and table 1). Overall, 60% of persons reporting with any symptoms tested positive for norovirus. There were no significant differences between patients and health care workers, or between persons in hospital associated outbreaks and nursing home outbreaks, although the highest proportion positives was found in the hospitalized patients reporting health complaints in association with an outbreak (85%), and HCW from nursing homes were least often NoV positive (50%).

Figure 1. Epicurve OB 1-7

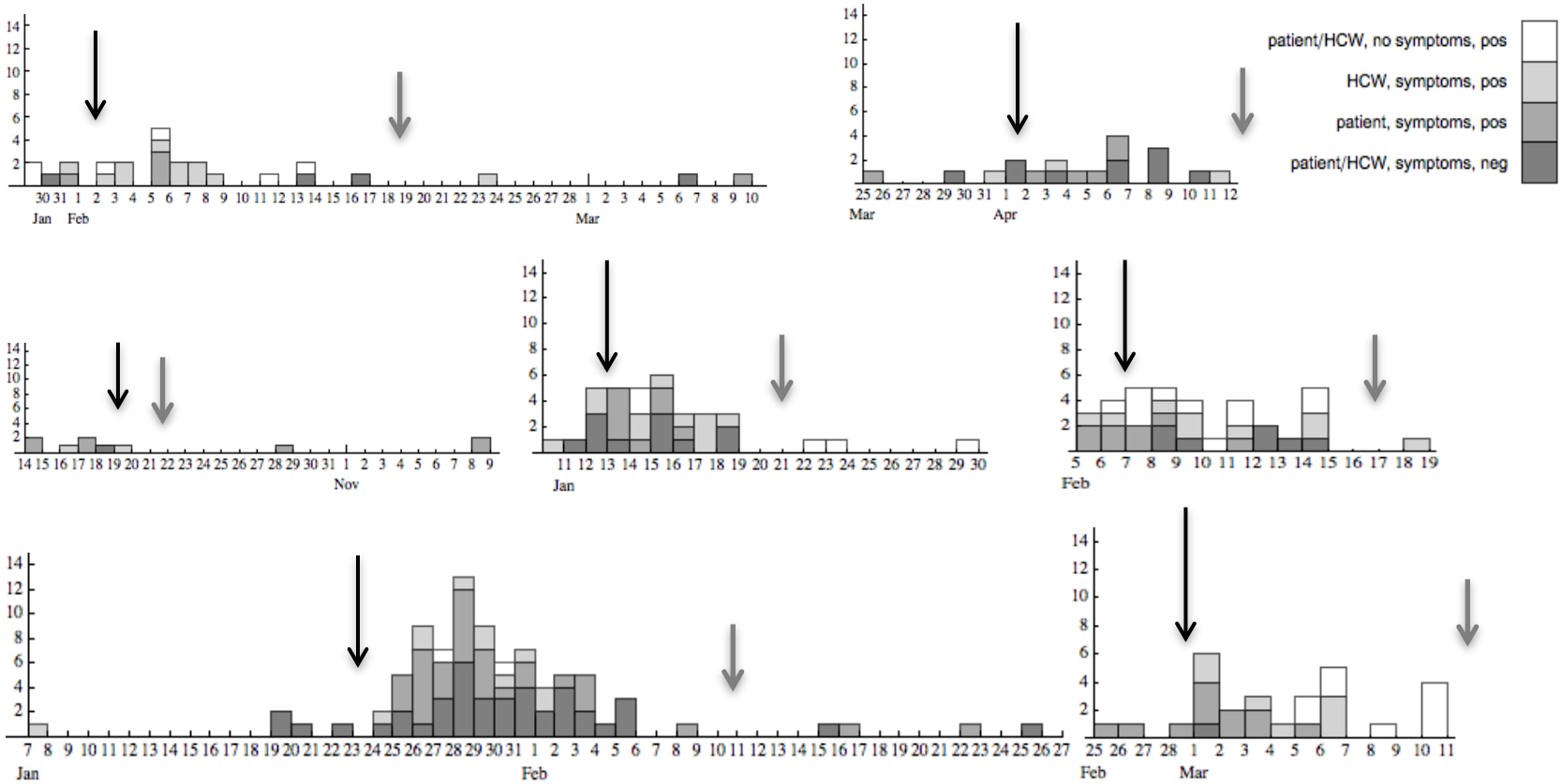

Figure 1. Outbreak (1-7), left to right are represented in the above figure. OB 4, 5 and 6 occurred in a somatic ward, while OB 7 occurred in a department for mentally impaired elderly. Negatively tested subjects with symptoms were attributed to the NoV outbreaks, since location, date and sampling were concurrent and no other agent (virus/ bacteria) was detected. The black arrows indicate the timing of ward closure. The end of the outbreak is represented by the grey arrows (by application the rule of 2 incubation period since the last case with symptoms). * Outside this stringent criterion there were still sporadic outliers with 100 % similar sequence in OB 1,5 and 6. (* not for all outliers sequencing was successful).

The proportion of NoV positive persons with symptoms was slightly higher for patients/residents (85%) than for HCW (76%), and did not differ significantly between settings.

The outbreaks had an average duration of 1-3 weeks, (the end of the outbreak is represented by application of the rule of 2 incubation periods since the last case with symptoms, indicated by grey arrows in figure 1). However, outside this stringent criterion there were still sporadic outliers with 100 % similar sequence in OB 1,5 and 6 (*not for all outliers sequencing was successful). Outbreaks in nursing homes had higher numbers of cases (mean nr positives 20 for hospital outbreaks versus 35 for nursing home outbreaks), (Table 1).

Table 1. Overview symptomatic & asymptomatic patients/ HCW versus positive/ negative

OB	Setting	Patiënts			HCW			Total		
		S+*	S-	AS+	**S+***	S-	AS+	**S+***	S-	AS+
1	H	**5**	0	0	**11**	4	4	**16**	4	4
2	H	**6**	2	2	**0**	9	0	**6**	11	2
3	H	**1**	1	0	**7**	7	0	**8**	8	0
4	NH	**7**	9	1	**11**	8	3	**18**	17	4
5	NH	**9**	2	6	**8**	6	4	**17**	8	10
6	NH	**18**	14	1	**6**	4	0	**24**	18	1
7	NH	**11**	0	0	**4**	4	4	**15**	4	4

* S+ = symptomatic, NoV positive; S- = symptomatic NoV negative; AS+ = asymptomatic, norovirus positive

The OB 2 and 3 were also different in the populations affected: OB 2 included only patients (adults and infants), and OB 3 included only HCWs and a single patient. In OB5 many asymptomatic NoV positive subjects were found, both among patients and

HCW. Two of the elderly subjects in OB5 died: the first person died 10 days after the start of the outbreak but tested negative for norovirus, while the second patient who died 18 days after the start of the outbreak tested positive for NoV.

Classification of clusters

Molecular sequencing of positive samples allowed classification of all the outbreaks by using the P2 domain as a molecular marker. In OB1, a small cluster of cases infected with a different norovirus was identified that was excluded from the analysis, as reported previously [14]. The remaining outbreaks consisted of single genotypes, and clusters were identified based on a (threshold) maximum of 2 nucleotides (NT) difference within outbreaks, based on a full fragment of the P2 domain (600 NT), thus considering that all the cases were linked [14] , (Including the outliers of each outbreak).

Control measures and cleaning

According to protocol, as soon as two or more subjects from the same ward tested positive for NoV, this was notified as an outbreak, resulting in ward closure and quarantine of infected individuals (figure 1, black arrows). Isolation of infected individuals was based upon detection of the virus; hygiene interventions (cleaning and disinfection of the whole department) were implemented at a later stage (2-4 weeks after detection of the first case). Information about hygiene interventions was only provided for the nursing home outbreaks. Regular staff was responsible for cleaning. During all observed outbreaks, any small accidents involving vomit or diarrhea were cleaned up immediately. Cleaning of the department took place approximately 2-3 weeks after detection of the first case (indicated by the black arrows in figure 1). The hospital wards were cleaned by an external company who did not log the exact dates. In OB6 and OB7, isolation of infected subjects was challenging because these wards housed patients with mental disorders. The patients in OB 4 and OB5 were physically disabled and were likely not aware of transmission contacts. Detailed on-site

observation revealed potentially unhygienic behavior of patients in all nursing home outbreaks (OB 4-7).

Sampling delay versus test outcome

A small number of PCR positive samples were retrieved from subjects on the day their symptoms started (day 0) consisting of 3 HCW and 4 patients and the negative cases (possibly including false negatives) were not included in the study. The majority of PCR positive samples were collected around 4-8 days post illness onset, while the majority of the negative samples were seen at illness onset, day 0. Overall, 60% of samples from symptomatic cases from whom date of illness onset was known tested positive, with a lower positivity rate during the first 4 days (50%) compared to days 5-8 (78%), ($p<0.05$).

The CT measurements showed a decrease between day 1- 4 post onset illness (increasing viral load), and a light increase over time (stable) and levels off beyond day 10. Note that these observations are based on small numbers of positives only (figure 2).

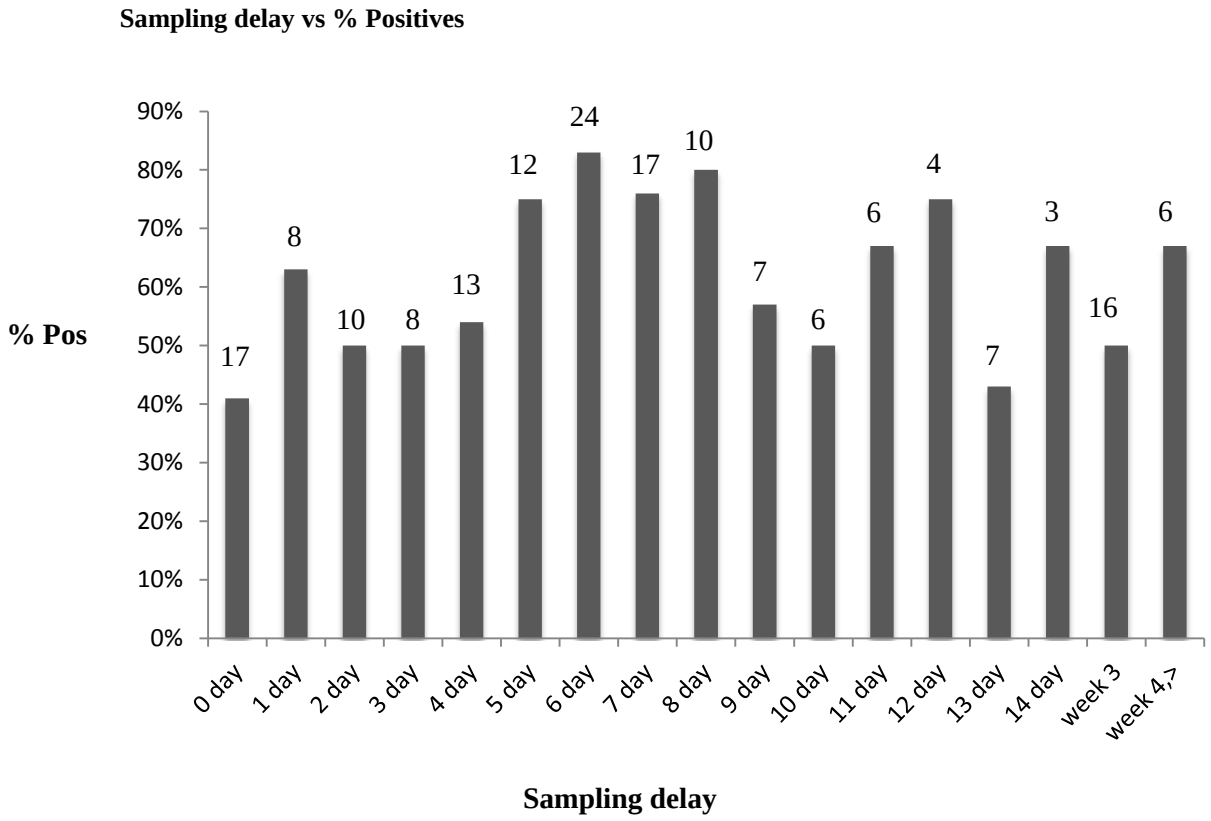

Figure 2. Sampling delay: time between onset illness and sampling are represented of 1-7 detected outbreaks against % Positives (the total # of cases are indicated on each bar). The majority of the cases were tested positive 4-8 days post onset illness. However, after 2 weeks positive cases were still detectable sporadically until day 30. With a sampling delay >8 days, a decrease of the number of positive subjects was observed.

Index cases

Transmission trees were used to identify possible index cases [8]. We also included persons with symptoms who tested negative in our selection of possible index cases. In total, 14 potential index cases were identified, of whom 4 were patients (in 4 outbreaks), and 10 HCW. Seven persons did not provide a questionnaire. The remaining possible index cases indicated that they thought they had contracted illness through contact with a case either in the hospital or nursing home (n=4), or at home (n = 1). The others did not provide any information indicating a possible source (Table 2). Based on this information, the most likely source of introduction was a patient or relative in 1 outbreak (OB2; the infant who was a likely index had a father who had

Table 2. Possible index cases and their demographics

Hospital outbreaks

NOZO	Potential index cases	Symptom status	Onset Illness	Contact with patients/ HCW, faeces, vomits	Sampling delay	PCR (CT)	Potential source, indicated by subjects
1072 (OB 1)	HCW	Vomiting (≥ 2/ day	30-01-2009	Unknown	13 days	Neg	No response
1078 (OB 1)	HCW	Diarrhea (2x days)	31-01-2009	Yes, in different wards	7 days	29	The virus/ infection was already introduced in the ward.
2524 (OB 1)	Patient	Asymptomatic	Sampling (4-02-2009)	Unknown	5 days	22	No response
3001 (OB 2)	Patient	Diarrhea and Vomiting (≥ 2x days)	25-03-2009	Contact with patients and HCW	12 days	Pos	Baby was admitted on 28-02, likely nosocomial infection. Father (of infant) also had symptoms.
1160 (OB 2)	HCW	Diarrhea (≥ 2/ day	29-03-2009	Unknown	16 days	Neg	Patient went on vacation and had already diarrhea.
1181 (OB 3)	HCW	Diarrhea, Vomiting (≥ 2x/ day)	14 Oct 2009	Yes and work on different wards.	11 days	Pos	No response
1255 (OB 3)	HCW	Vomiting (≥ 2x /day)	14 Oct 2009	Unknown	11 days	Pos	Boyfriend had symptoms afterwards

Nursing home outbreaks

NOZO	Potential index cases	Symptom status	Onset Illness	Contact with patients/ HCW, faeces, vomits	Sampling delay	PCR (CT)	Potential source, indicated by subjects
11030 (OB 5)	Dietician (HCW)	Diarrhea (≥ 2/ day	10-01-2010	Yes and work on different dept.	16 days	26	Had ill family members (8-13 Jan. 2010) Indicate that she introduced the virus to the nursing home, since she was the first case with symptoms detected
11026 (OB 5)	Dietician (HCW)	Diarrhea (≥ 2/ day	11-01-2010	Yes and work on different dept.	17 days	Neg	No response
11072 (OB 5)	Section lead	Diarrhea (≥ 2/ day	11-01-2010	Unknown	11 days	Neg	No response
11196 (OB 6)	Trainee Nurse	Diarrhea (≥ 2/ day	5-02-2010	Yes and work on different dept.	9 days	30.7	The same department where she was working
11123 (OB 6)	Patient	Diarrhea (≥ 2/ day	5-02-2010	NA	8 days	24.7	Another admitted patient on the department
11288 (OB 7)	HCW		19-01-2010	Yes and work on different dept.	NA	Not tested	No response
11371 (OB 7)	Patient		25-02-2010	NA	17 day	28	No response

Table 2. Overview of possible index cases with their demographics representing a more realistic view of the introduction of the virus within the health care setting of OB 1-7 based on the provided demographics and serial sampling. Almost all of the index cases reported symptoms and the majority of them were HCW who had possibly worked on multiple departments, having contact with several occupants. A quite mixed pattern of sampling delay versus PCR positives was observed among these index cases (long and short intervals. The history before the outbreak started was poorly reported for OB 1,2,3,6 and 7).

symptoms of gastroenteritis at the same time). For three outbreaks, based on the response of initial cases the index may have been missed, reflecting late detection (OB's 1, 6 and 7). Based on the combined transmission analysis, in OB 4 a HCW (dietician) was the most probable source of the introduction of the virus within the department. This HCW was the first subject who tested positive for norovirus within the affected ward. She visited several wards, as well as other nursing homes in the region. In the questionnaire, this person noted being the first person with gastroenteritis symptoms in the department, and having household members who had experienced similar gastroenteritis symptoms. For OB3 and 5 no information was provided due to non-response or incomplete questionnaires. For OB4 and OB5, sporadic cases had been observed prior to the index cases, according to department logs. In the period before OB 4 and OB 5 were recognized, there had been an outbreak of norovirus in a different department in the same institution.

Conclusions and discussion

Our results confirm that with currently attainable levels of infection control, norovirus transmission in healthcare settings cannot be completely prevented. We believe that thorough understanding of the introduction and transmission of the virus can help in setting guidelines for effectively reducing the spread of NoV. We have shown that transmission analysis including enhanced molecular typing allows unambiguous identification of clusters. Combining these transmission studies with questionnaire surveys incidentally did augment understanding of how the virus was introduced, but proved very challenging. Despite extensive investigation, only in two outbreaks, a most probable source of introduction was identified. The investigation suggested that – despite our efforts for enhanced awareness throughout the study, initial cases may have been missed, as initial patients for three outbreaks indicated that they had been in contact with a symptomatic person at work. As this could be confirmed later by a detailed review of the department logs, one of the lessons is to include such review as part of outbreak investigations. Currently such context studies are not part of the protocol in the participating institutions. Previous studies have demonstrated a diverse pattern of index cases: in 30 nosocomial outbreaks with person to person

transmission, at least 20 (67 %) had a patient as a likely index case [16]. In another recent study, it was concluded that out of 5 nosocomial outbreaks, at least 4 started from a patient [17]. However, HCWs introducing norovirus have also been reported [16, 18]. We found that enhanced sampling during outbreaks among patients and residents almost doubled the number of recognized norovirus infected persons, some of whom remained without symptoms. Therefore, a question is how reliably the data from standard outbreak investigations may allow identification of defining index cases. An aspect that should be considered in transmission studies is the opportunity of contact between subjects. As HCWs work in shifts, possibly 8 hours in duration, they are available for contacts for about 1/3 of the 24 hours of any day, while patients or residents are usually present for the complete 24 hour period [8, 19, 20]. We have found that symptomatic cases, particularly patients with diarrhea, cause more transmission than asymptomatic HCWs [8]: symptomatic patients often act as key transmitters during outbreaks. This is also consistent with reports that the dominant route of transmission during outbreaks is patient-to-patient transmission followed by patient to HCW transmission [20].

Introduction of norovirus into a health care institution may depend on various factors. Compared to patients or residents, HCWs have many (more) opportunities to introduce or spread the virus: they are usually involved in preparation of foods as well as feeding of residents, have physical contacts with excreta and patients, and they visit other departments and health care centers. Therefore, they likely occupy a central position in the contact network for virus transmission. Since most norovirus outbreaks occur during the winter (the peak season for norovirus outbreaks), introduction from the community should be a factor of concern particularly during this season.[21]. Once index cases are recognized, special attention should be given to the first generation of secondary infections (transmission from the index cases to the other subjects), since this is a crucial moment for interruption, and because the first week of a norovirus outbreak often shows the highest intensity of transmission [8]. For an optimal detection of these cases, results of RT-PCR should be interpreted with caution. During our investigation, only 60% of persons reporting symptoms tested positive. Although we cannot exclude false attribution of cases to the outbreak, but this lack of PCR positives could also point at sensitivity issues. When reviewing the data for persons for whom date of onset was known, we observed that sampling too

early during outbreaks may result in negative PCR while targeting sampling between 5-8 days post onset illness can increase the number of positive results.

A study evaluating effect of control measures implemented during outbreaks in nursing homes in The Netherlands found that timeliness of interventions (within 3 days post first case notification) was the only factor with a notable impact [22]. Despite isolation of cases soon after detection of NoV, transmission was still seen in the outbreaks studied here, in agreement with published findings [19, 22]. Realistically, even if the first case should be identified within a few days, intervention would be already too late to completely stop transmission. Therefore, rapid and efficient diagnostic testing is important, to routinely survey patients at admission and HCW at regular intervals, particularly during the peak season (winter). It is also important for infection prevention staff to create awareness among HCWs of their role in norovirus introduction and transmission. It is useful to reiterate that even when one is too late, quarantining may be applied to prevent the outbreak to grow even further beyond affected wards.

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Chronic Shedders as Reservoir for Nosocomial Transmission of Norovirus

Chapter 8

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Abstract

Norovirus infection in immunocompromised patients may lead to prolonged norovirus shedding. Here, we demonstrate involvement of three chronic shedders in hospital outbreaks. Combined epidemiological and molecular evidence suggests that in one case NoV transmission occurred at least 17 days after the first diagnosis.

Introduction

Norovirus (NoV) is a leading cause of acute gastro-enteritis affecting people of all age groups (1, 3). Outbreaks with NoV occur often and worldwide (7). In the Netherlands, large numbers of outbreaks are reported each winter, particularly from health care institutions (6). In a systematic evaluation of newly diagnosed patients with norovirus in a large tertiary care hospital, we found that nosocomial norovirus transmission is common, and may lead to chronic infection, disease and shedding in at least 6% of patients (1). The case histories of the chronic patients showed various underlying illnesses resulting in impaired immunity followed by prolonged NoV shedding, in some cases for periods longer than 1 year (5). The question arose whether these chronic shedders were possible sources for nosocomial infections within the hospital setting, also after they had been infected for a number of weeks. Because NoV cannot be cultured *in vitro* (2), it has remained impossible to assess whether the viruses shed by such patients are still infectious. The finding that noroviruses evolved within chronic patients suggested that detailed molecular virological data in combination with epidemiological data could be used to track possible routes of NoV transmission within the hospital (9, 10).

Epidemiological records for all NoV positive patients diagnosed between 2005 and 2007 were retrieved from the Erasmus Medical Center (EMC) data bank, including admission dates, sampling dates and departments. Fecal samples associated with these cases had been stored at -80°C. Patient samples were sequenced as previously described (9). Briefly, the P2 domain of the ORF2 with a length of approximately 700 nucleotides was sequenced in both directions using the ABI Prism BigDye Terminator version 3.0 ready reaction cycle sequencing kit. Strain sequences from patients with chronic NoV had been described previously (5). Norovirus positive patients hospitalized in the same period (defined as six months before to six months after the first sampling of all chronic shedders) were selected, and their stool samples were used for analyses. Only strains that were unique and showing clustering with the chronic patients were included. This selection was made to represent background diversity of norovirus strains circulating within the hospital. The strain sequences circulating in the community were represented by strains diagnosed at day 0 after

admission. To identify patients who may have been nosocomially infected by chronic shedders (5), strain sequences were obtained from patients that were routinely hospitalized with various disorders excluding NoV as a cause. The obtained sequences were identified on bases of 100 % identity to sequences previously obtained from chronic shedders over a minimum fragment length of 600 nucleotides. The sequences were subsequently analyzed using TREECON For Windows (8) with the neighbor joining method (single rooted) followed by bootstrapping. (Fig. 1)

During the study period, we found three molecular clusters containing sequences of patients who had been recognized as chronic shedders patients and other hospitalized patients; two clusters consisted of genotype GII.4-2006a and one of GII.3 strains (Fig. 1, Table 1). Chronic patient 6 (numbering corresponding with reference (5)) was admitted to the hospital multiple times, while chronic patients 4 and 8 stayed in the hospital, mainly in the same location during their norovirus infections. They were sampled and tested for NoV repeatedly during their admissions or visits, and sequences identical to theirs were detected among other admitted patients. Based on molecular information combined with demographic data the most probable direction of transmission was assumed to be from the chronic patients to other hospitalized patients. The transmissions between chronic patients 4 and 6, and the other patients in the two GII.4 clusters occurred shortly after the chronic patients were first diagnosed (sequence tree not shown). In the GII.3 cluster, transmission was detected both shortly after initial diagnosis of the chronic patient (involving at least five other patients), and also after a longer interval (involving one patient; NT23) (Fig. 1). The patients who were infected during the first week of this hospital outbreak, all shared identical sequences in the genomic region analyzed. The sequence of the NoV strain detected from the second sample drawn on day 17 in chronic patient 8 showed one nucleotide difference compared to the strain detected on day 0, and was identical to that of patient NT23, whose onset of disease occurred 20 days after the onset of disease of chronic patient 8. This strongly indicates that this patient, a 6-month-old ex-premature child with symptomatic nosocomial infection, was infected by chronic patient 8, at least 17 days after the first time norovirus was detected in patient 8 (day 0). The NT patients were hospitalized concurrently with patient 8 except for NT 19, 20, 22; they were admitted three to six days before chronic patient 8, followed by NT 23 who had been admitted almost two months earlier than chronic patient 8 as indicated by the blue bars in figure 1. Two other clusters that included chronic shedders remained

Table 1. Transmission of NoV from three chronically infected patients.

	Cluster size # patients [a]	**Available samples**	**NoV diagnosis of the chronic patients after # days**	**Clinical symptoms chronic patients**	**Transmission delay # days** [b]	**Genotype**
1	5	**Chr. Pat. 4** and 3 NT Pat.	3	Admitted with chronic diarrhea	4	GII.4-2006a
2	3	**Chr. Pat. 6** and 1 NT Pat.	13	Admitted with infant regurgitation	4	GII.4-2006a
3	9	**Chr. Pat. 8** and 6 NT Pat.	2	Admitted with diarrhea and vomiting,	2, 3, 6, 7 and 17	GII.3

[a] Each cluster contains one chronic patient (indicated as Chr. Pat. 4, 6 and 8) and other hospitalized patients indicated as NT (norovirus transmission) patients.

[b] Transmission delay was defined by the number of days between the date of diagnosis of the chronic patients and the onset of illness of the other patients in the cluster.

GII.3 NoV transmission from chronic patient 8 based on identical P2 domains

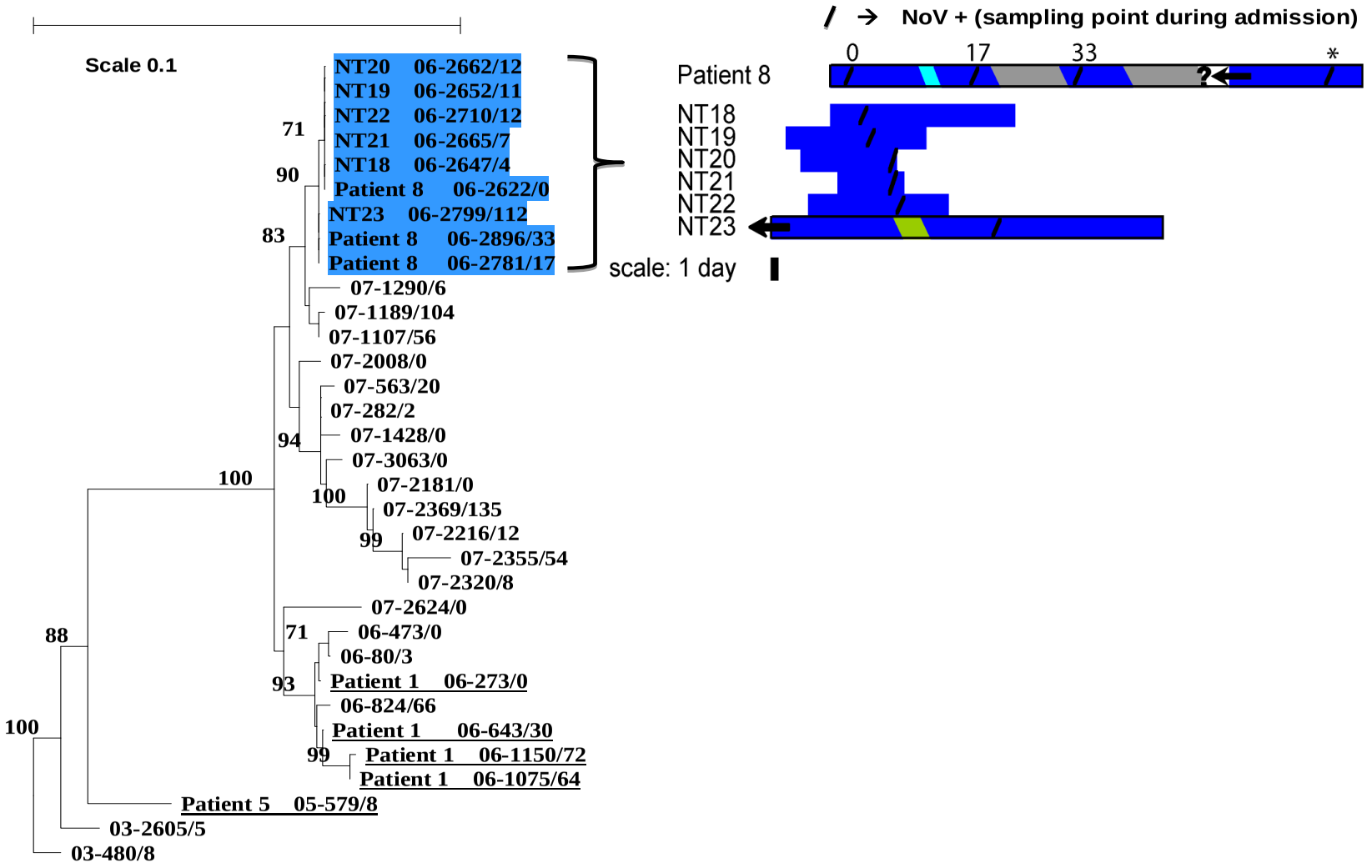

Figure 1.

Phylogenetic tree (TREECON for Windows) representing the sequences of the P2 domain of the GII.3 strains (left), analyzed with the neighbor-joining method followed by bootstrap analysis using 100 pseudoreplicates. Each strain is labeled with the patient identification code and XX-YYYY/Z, where XX is the year (e.g., 07 is 2007), YYYY is the unique case code, and Z is the time of diagnosis by the number of days after admission. At the right, the transmission scheme of cluster 3 is shown. In this scheme, detailed information describing the admission times and locations of the patients involved in the outbreak with patient 8 are shown. The chronically infected patients included in this tree are patients 1, 5, and 8. The colors (green, blue, and light blue) in the scheme indicate different departments where the patients were localized during their admission; gray indicates that the patient was discharged from the hospital. The black diagonal slashes show the sampling points during admission, while the sharp black arrow with a question mark indicates that no admission and discharge information was available. The blunt black arrow shows the admission date of NT23 (almost 2 months before that of chronic patient 8). The asterisk indicates that there was not enough sample (feces) available to perform norovirus diagnosis, while the patient had diarrhea or symptoms.

unresolved with respect to the direction of transmission, because all cases were diagnosed within a few days. However, patient 4 already had chronic diarrhea prior to hospitalization, which was resistant to treatment and coincided with chronic shedding of NoV. Therefore, it is plausible that this is a second example of transmission from a chronic shedder. Evidence was most convincing for patient number 8, for whom the second sample (taken at day 17) showed a unique mutation that was identified in another patient hospitalized in the same ward. As this sequence was unique in the entire dataset, a link with the chronic shedder is highly likely. However, sources of NoV in the hospital may vary from patients to staff, contaminated environments and food items and despite extensive outbreak investigations, the exact modes of transmission often remain unclear. This study, however, shows that chronically infected patients may contribute to the spread of NoV in hospitals. To our knowledge this is the first study that provides evidence for this hypothesis and points at an important aspect of infection control: contrary to earlier beliefs, patients who had NoV illness may shed the viruses for weeks, and recent data suggest that chronic shedding is relatively common in persons with impaired immune functions who contract the illness. Given the high incidence of NoV infections and the increasing size of the population that is immunocompromised, this problem is likely to increase in the years to come (4). Therefore, as part of infection control policy in the hospital, the possible contribution of such patients to nosocomial spread should be considered.

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Summary and General Discussion

Chapter 9

Summary

Norovirus has become an important agent of gastroenteritis worldwide, including the Netherlands, causing large and small outbreaks in various health care settings and age groups. The high numbers of reported outbreaks in the Netherlands, with a substantial public health impact, have raised questions about control of norovirus outbreaks in high-risk settings. Previous studies have enhanced the understanding of the clinical impact and the epidemiology of the virus, but the knowledge of transmission remains limited [1, 2].

The focus of this PhD project was to enhance insights into the understanding of the introduction and the spread of the virus during outbreaks, using a range of methods for molecular typing and epidemiological studies, to improve the evidence basis for targeted prevention strategies.

Norovirus outbreaks are difficult to control and will remain a challenge to public health in the years to come, particularly in nursing homes and similar healthcare settings. However, implementation of appropriate infection control measures is critical to controlling an ongoing norovirus outbreak. Stringent infection control practices are necessary for closed facilities including healthcare settings where the close proximity of residents may facilitate rapid spread of Noroviruses.

Unfortunately, our understanding of this important area is significantly deficient and requires further study. Methods that allow identification of transmission links, both at the level of outbreaks and at the level of individual cases may greatly enhance our understanding of the ecology of these viral pathogens.

The impact of norovirus transmission in nursing homes and tertiary care hospital environments

Norovirus transmission has been reported in many community care-based settings, and possible modes of transmission and sources of introduction of these viruses have been studied [3]. Since norovirus is highly contagious, infected symptomatic individuals within health care settings can easily transmit the virus among both patients and staff through infectious excreta, via person to person contact and contaminated surfaces [4].

Previous studies in residential settings revealed a high incidence of non-suspected viral gastroenteritis, where norovirus was found in 86 % of the studied outbreaks [5]. Another study performed in the Netherlands showed that about 70 % of all the outbreaks caused by norovirus occurred in nursing homes and homes for the elderly and predominantly consisted of person to person transmission [6].

Our findings confirm that nosocomial norovirus outbreaks are common, usually requiring closure of the affected department. Among the prospectively monitored outbreaks in this study, nursing homes were in majority and were greater in size compared with the hospital outbreaks (sukhrie et al, unpublished).

Further to our understanding, once the virus is introduced in these settings with high contact rates, it can easily start an outbreak and subsequently cause serious disruption of the working routine. Patients with norovirus infections require additional care and they may infect health care workers who deal with these infectious patients on a daily basis. In addition, the workload for the health care workers increases further due to sick leave of colleagues. Recent studies have suggested that exposure of immuno-compromised patients and elderly residents to norovirus could result in fatal infections [7, 8].

Another important consequence is closure of the affected department; temporary stop of admissions, and implementation of control measures, causing substantial financial losses during norovirus outbreaks. These findings implicate that advanced protocols and control measures are needed to eliminate transmission in an early stage of circulation of the pathogen. As recently demonstrated, application of control measures

in the earliest stage during an outbreak can help to prevent secondary infections and control of the outbreak [9]

Currently there are many guidelines and outbreak management protocols in use at health care institutions worldwide aiming to prevent and control norovirus outbreaks in high risk settings [10]. These protocols focus on hygiene interventions: adequate hand hygiene for suspected norovirus cases and any contacts, including staff; isolation gowns and gloves for contact with incontinent persons during outbreaks, and at every moment there is risk of contamination with infected vomitus or feces. Furthermore, quarantine measures may be taken: such as use of private rooms or cohorting to segregate suspected norovirus cases, and finally, disinfection measures such as regular cleaning of contaminated surfaces. Despite knowledge of these guidelines, norovirus outbreaks seem to be increasing worldwide, suggesting that current application of these control measures is insufficient in reducing norovirus transmission [11]. As the focus of intervention measures is on limiting transmission during outbreaks, there is limited understanding of how the virus is introduced into healthcare settings. It is likely that the high incidence of infection, including many asymptomatic infections, causes the virus to be easily introduced into nursing homes and tertiary care hospitals, due to the continuous exchange and admissions of patients and visitors. Once the virus has been introduced, mitigating or preventing transmission is difficult and usually too late: even the best hand washing procedures and eradication protocols may fail to curb an outbreak. On the other hand, hygiene interventions remain important in limiting the spread of the virus and thus the impact of an outbreak [10].

Routine surveillance and tackling introductions and (nosocomial) transmissions of noroviruses

Outbreaks of norovirus often remain undiscovered and the source of the virus usually remains unknown. By the time norovirus infection is diagnosed, transmission is already ongoing and several subjects may have contracted the virus. Currently, emphasis is on enforcing increased hygiene during norovirus outbreaks, but sources of introduction, nosocomial infection and transmission are rarely studied or described.

Our studies indicate that introduction of the virus is a key element of transmission which should be included into measures for reducing virus spread (sukhrie et al, unpublished) [4, 12].

Our observations also indicate that norovirus screening and detection in healthcare settings is suboptimal, which may lead to increased diagnostic- and treatment costs, as well as complications in frail patients with immuno-suppression. In one of our studies we demonstrated that systematic screening of patients whose fecal samples were submitted to the laboratory (instead of testing at clinician request only), almost doubled the number of infections detected [13]. In characterization of another prospective outbreak we found that enhanced sampling yielded a 232 % increase in cases, including asymptomatic patients, symptomatic and asymptomatic health care workers [14]. These findings clearly indicate that norovirus infections are frequently missed.

Using a database of retrospective outbreak data in a tertiary care hospital documenting nosocomial spread of the virus, it was shown that five of the 14 clusters involved at least one outpatient, thus indicating that targeting these outpatients may improve the infection control measures and perhaps limit introduction and (nosocomial) norovirus transmission [3].

Therefore, demographics, behaviors and additional contextual data of infected subjects in outbreaks should be reviewed to study their roles in the transmission network. However, collection of such data proved challenging, particularly for HCW, since very little information is available. Lack of knowledge about the source of the virus in many of the studied outbreaks is of concern, since we can only speculate about the effectiveness of measures aimed at preventing virus introduction.

Norovirus sources are difficult to characterize and it often remains unclear where the outbreak strain originated. Since noroviruses are widely circulating in the community, this is always a likely reservoir. For instance, as we have seen during our studies, strains from patients exclusively visiting the emergency unit were sometimes identical to strains that later caused an outbreak in the same hospital, based on comparison of P2 domains (600 bp) [3]. This study demonstrates that sporadic strains from the community can become epidemic and cause outbreaks. Therefore, such introductions of sporadic strains into a nursing home or hospital should be investigated in more detail. The means of introduction is also a factor of concern, with emphasis on

purpose of contact such as occasional visitors, or healthcare workers with daily access to the hospital or nursing home. In one of our recent studies we demonstrated that norovirus might be introduced by both HCWs and patients (Sukhrie et al, unpublished). Based on their self-reported data we could conclude that they might have contracted the virus outside the institution, e.g. sick family members. Our studies also show, however, that in many instances, the source of (introduction) of the virus cannot be unambiguously identified.

Once introduced, noroviruses spreads particularly well in healthcare settings, leading to nosocomial infections. A special impact is on immunocompromised patients that may not be able to clear norovirus and shed norovirus for a prolonged period of time, as we have seen in one of our studies. These shedders may constitute an important role within the health care setting and may act as reservoir for norovirus [15]. Therefore, contact with these chronic shedders requires special attention.

Transmission of noroviruses from an ongoing outbreak may also occur where for instance health care workers working on an outbreak unit may concurrently service a non-outbreak unit. Recently a study demonstrated that health care workers employed at different facilities introduced the virus into at least three of their other working locations [16]. Thus, HCW are likely to play an important role in transmission because of their mobility, their contacts with patients or residents and various extramural environments. For this reason, we surmise that HCW may be important as a link between cases even though we have documented that their probability of transmission is smaller than that of patients or residents. Within the contact network, their higher connectedness, compared to patients or residents, may be important for carrying an outbreak across environment boundaries. Both health care workers and patients can act as key transmitters and cause secondary cases. Superspreaders, who cause more secondary cases than most others as recognized from our possible transmission trees, also deserve special attention, because knowledge of any factors that render an infectious subject a superspreader, may help in targeting more effective intervention procedures.

Routine testing of norovirus, particularly subjects with symptoms may help to obtain insights into the introduction of the virus either by symptomatic or asymptomatic cases even though within outbreaks, asymptomatic cases seemed not as effective in transmitting the virus as symptomatic cases [14]. Additional information about the

mechanisms involved in transmission may be obtained by assessing questionnaires collected during an outbreak, which may provide self-reported case histories, systematically assessing possible sources (other patients, personnel, visitors, environment, food etc.) and transmission routes.

We have shown that the combination of transmission analysis and contextual data obtained from questionnaires revealed important clues to identify index cases in the studied outbreaks. In view of the crucial role of infected health care workers we noticed that the sick leave for this category can range from 48 hours up to 6 days. It is important to note that virus shedding may last up to 60 days or more, while symptoms last a week or less. However, based on studies reported in this thesis, transmission from an asymptomatic health care worker is not likely. Therefore, HCW returning to work as soon as their symptoms have disappeared, should not cause additional cases, as long as they comply with hygiene protocols [4]. On the other hand, symptomatic patients have the largest contribution to virus transmission during nosocomial outbreaks. Since all the prospectively monitored outbreaks occurred during the winter season, screening of patients at admission particularly during the winter combined with rapid strain typing, and (anonymized) linking of illness reports from healthcare workers to those in patients may contribute to earlier detection of clusters. Thus, strategies can be designed to decrease the impact of specific introductions, and to develop evidence-based recommendations for the prevention and control of norovirus outbreaks in hospitals and nursing homes.

Rapid testing and combination with Real Time PCR

Due to the rapid transmission of noroviruses, rapid control measures are needed for infected patients and health care workers to break the chain of transmission. Therefore, quick diagnosis of noroviruses is crucial, requiring highly sensitive, and in particular, rapid testing. If testing takes too long and if noroviruses are missed during testing, unidentified infectious subjects might be able to rapidly transmit the virus resulting in a growing outbreak.

Sensitive testing currently implies real time PCR (RT-PCR), which requires highly trained personnel, and is difficult to implement in nursing homes and homes for the elderly. Such "gold standard" assays are expensive and often slow. Efforts are underway to decrease the time to diagnosis with RT-PCR, but currently the procedure for norovirus still takes too long, particularly when tests cannot be done onsite. We have seen in outbreaks that nursing homes do not have a point-of-care test: and in most of the cases samples are sent for testing to external laboratories, resulting in a delay of approximately one week before the results are reported (Sukhrie et al, unpublished).

Therefore, effective and rapid testing is instrumental in initiating control measures as early as possible. A bedside test or a point of care test is appropriate in settings with less comprehensive laboratory facilities, but highly trained personnel. Such tests are less sensitive than RT-PCR, which may cause false negatives based on rapid testing. However, different evaluation studies have shown that these methods are more successful in detecting the GII variants, rather than the GI strains [17-19]. Since the GII viruses cause the majority of the outbreaks in these settings, balancing the slower but sensitive and specific RT-PCR test with the rapid but less sensitive and specific bed side test could be a viable solution to diagnose norovirus more rapidly.

A competing challenge is the amount of testing during norovirus outbreaks, and testing for routine screening. Since numbers of norovirus cases are increasing, a less labour intensive and rapid test is useful. A recently developed BLEIA (bioluminescent enzyme immunoassay) test, which is operated by an automated device and does not involve complicated procedures, can be used at hospitals and clinical laboratories to rapidly test large numbers of samples. [20].

Besides detection of the virus, typing should also be part of routine outbreak investigations, to help identify clusters. Recently it was demonstrated that applying sequence typing during an outbreak within a short period of time revealed four different strains, indicating multiple introductions and limited nosocomial transmission [21]. Such findings can be very helpful for decision makers and health professionals to guide control measures. For instance, when there is no nosocomial transmission but only multiple introductions, closing the facility is not necessary. During follow-up specific measures could be implemented to characterize these introductions. For reliable strain differentiation, P2 domain sequence typing is

required, to avoid misclassification of strains of the same genotype, particularly to recognize pseudo outbreaks during outbreak surveillance and multiple introductions at the same time of the same strain [12].

Molecular characterization and accumulation of mutations of the Norovirus genome in the context of tracking transmission

Molecular characterization for norovirus is widely used for diagnostics. As described earlier, mapping of clusters and identification of nosocomial transmission are usually based on epidemiological data, like concurrent location and timing [3, 12]. However, we have shown that using only time and location, and norovirus diagnosis, misclassification of outbreaks and cluster mapping can occur [12].

To unambiguously characterize transmission during outbreaks, a combination of detailed molecular and epidemiological data is required [3]. We have demonstrated that understanding of these combined observations (Molecular and Epidemiological data) provides a scientific basis for transmission tracking and consequent control measures [3, 12, 14, 15]. Molecular linking of cases, and outbreaks, may be used as a tool to unravel the ecology of pathogens, but understanding the rate of genetic change is essential to design evidence-based sequence typing for use in epidemiological studies. Noroviruses have an RNA genome in which mutations accumulate at a high rate [22]. Since a person infected with a single infectious particle may shed many millions of progeny viruses, the majority of these are likely to have one or more mutations compared to the parent virus. Some of these altered sequences may be successfully transmitted. Studies have reported the rapid change in the P2 domain expressing the outermost capsid protein, which can interact with both carbohydrates (CHOs) and antibodies [23, 24]. Recurrent emergence of new epidemic norovirus strains can be expected with norovirus antigenic drift, which allows for noroviruses to evade the human herd immunity [25].

Molecular analysis suggests that norovirus evolution is driven by immune selection but infections in healthy individuals result only in limited and short term immunity [26]. Therefore, these evolutionary changes seem likely to occur in people with

compromised health (immunocompromised, elderly), where long term shedding and substantial changes in viral genomes have been described [27, 28].

Understanding the rate of change is essential to design evidence-based sequence typing for use in epidemiological studies, [3, 12, 29]. We found that P2 domain sequence changes do occur during outbreaks but found a maximum of two nucleotide changes within a limited time frame of the studied outbreaks [12] Some of these contained mutations leading to amino acid changes possibly involved in the receptor binding patterns of the virus [12, 30].

As previously suggested, recombination may an important mechanism for the emergence of new strains including interchange of genomic regions (P1 and P2 domain) and exchange of antigenic elements [31, 32]. During our transmission studies, no mechanisms of recombination and exceptional interchanges of the gene encoding the partial capsid protein were observed. The circulation of Norovirus in the general population and their sustained circulation in selected risk groups may contribute to a reservoir generating for new variants, which in turn could cause new outbreaks. If that is the case, interventions in these risk groups could potentially influence the (global) epidemiology of Norovirus. Possibly, further classification of strains using deep sequencing during outbreaks and regular screening from the general population could help to identify minor and major variants, as well as epidemic variants and the possible direction of transmission [33, 34] .

Our understanding of the evolutionary mechanisms is still developing: predominant use of the P2 domain as a marker in tracking transmissions will likely result in the discovery of noteworthy mutations within this domain. The focus of sequencing should be extended by focusing advanced methods providing higher resolution P2 domain sequence data. For instance distinguishing minor strains from the major strains during outbreaks per sampled individual or sequencing larger parts of the genome (or the whole genome) may help in identifying specific routes of person-to-person transmission.

Concluding remarks and future perspectives

To understand transmission pathways of norovirus between the general population and health care setting, and within health care settings, a standardized approach to outbreak investigations, using validated methods is needed. Importantly, collecting data on the extent of transmission of norovirus during outbreaks, factors contributing to their introduction and spread, and data on diversity of the genomes of viruses shed by people over subsequent chains of transmission can be used to identify transmission networks and possibly lead the way to control measures as discussed previously.

However, collection of such data is quite challenging in nursing homes and elderly homes with limited resources and personnel. At the start of the study, the research nurse gave presentations and distributed flyers explaining the study to health care workers in each of the involved institutions. The studies had been endorsed by the management, and a member of our team supported HCW in administration of questionnaires and sampling logistics. Despite this, we have seen that even performing prospective controlled outbreak studies was challenging in these settings, resulting in many non-responders and generally limited interest for such research. In part this can be explained by the workload: as during outbreaks, the health care workers and patients are under strain from worsening conditions and challenges imposed by outbreak protocols, such as intensified hygienic measures. We have also experienced that the transition from a normal routine to an outbreak routine can be quite labor intensive due to sick leave of colleagues and application of control measures. This may also have motivated the decisions of patients and health care workers to withdraw from the study.

Creating awareness is an essential start, perhaps launching effective campaigns about the consequences of norovirus outbreaks in the home environment, rather than focusing on particular settings. This should convince institutions and health care workers of the importance of norovirus transmission research in this field.

A relatively straightforward routine practice could be screening of health care workers and patients sharing wards with norovirus cases as part of the outbreak investigations, with storing of samples for analysis if needed, for instance if an outbreak persists. This should be accompanied by a standardized and short questionnaire to obtain demographics and behavioral notes of patients diagnosed with norovirus symptoms on routine basis. These demographics should at least include the food consumed and

where that person might have contracted the virus (household members, friends, and contacts). Our work shows that particular emphasis is needed to gain insights on the origin of the outbreaks, in particular where there is additional knowledge from the self-reported questionnaires focusing on context e.g. personal hygiene and physical contact with other occupants. Similar systematic or behavioral studies should be made of the contact behaviors of health care workers and their roles in the transmission chain of noroviruses. Such information can provide critical support for decision making for infection control staff, municipal health services and food safety inspectorates. We have seen that the majority of the outbreaks in this study(5 out of 7) were caused by the GII.4 strain, as this is the most common circulating strain attributed to major outbreaks, particularly in health care settings. Work done in a European research network coordinated by National Institute for Public Health and the environment in the Netherlands has also shown that in the past 12 years four successive GII4 variants emerged and caused worldwide epidemics [35, 36]. Therefore, particular focus on this genotype variant is highly required in these settings. It is also important to focus on the understanding of the rate of genetic changes, which is essential to design evidence-based sequence typing for use in epidemiological studies. In addition to collecting data on onset of symptoms and locations of cases, fecal samples should be collected whenever possible. P2 domain linking should be a routine in outbreak characterization combined with collecting self-reported contextual data of suspected individuals (health care workers and patients). However, further additional analysis of this domain is needed with regard to sequence changes in a given outbreak among strains of the same origin and within any affected individual in a subsequent time frame. We have shown that within outbreaks, minor variation in the P2 domain may be found, and that the probability of finding sequence changes increases with time. To better characterize P2 domain variation within outbreaks prospective follow-up studies on infected individuals (health care workers and patients) are required, to ultimately validate the cut-off we have suggested for current analysis and strain segregation. During our study we could not reach the targeted numbers of outbreaks due to limited occurrence of outbreaks within the study period. There were lots of cases during the outbreaks that tested negative although they were screened within the outbreak criteria and displayed norovirus symptoms resulting in limited numbers of samples[14]. As previously mentioned, more samples and advanced sequencing (next generation sequencing) could be very useful to

systematically validate the P2 domain and perhaps other regions of the norovirus genome for transmission analysis [33, 34]. In conclusion, this study showed that current techniques for preventing the spread of norovirus in health care settings are inadequate due to the rapid nature of virus transmission. We recommend both further molecular analysis to identify sources of infection to be made routine, as well as development of rapid diagnostic tools to reduce the incidence and spread of infection in these vulnerable groups of people.

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Samenvatting

Dankwoord (volgt nog)

Curriculum Vitae

Publicaties

PhD Portfolio

Samenvatting

Norovirus (NoV)-infecties zijn de meest voorkomende oorzaak van buikgriep bij personen van alle leeftijden. Daarnaast zijn NoV's berucht als veroorzaker van uitbraken in zorginstellingen, op cruiseschepen en in restaurants. Dit proefschrift beschrijft onderzoek gedaan van 2008 tot en met 2012 in zorginstellingen in de regio Rotterdam, met als doel beter inzicht te krijgen in de introductie en verspreiding van NoV als basis voor het ontwikkelen van *evidence-based* richtlijnen voor bestrijding.

Het doel van deze studie was om systematisch onderzoek te doen naar bronnen van infecties en contacten tussen geïnfecteerde patiënten teneinde inzicht te krijgen in de herkomst van de virussen en de wijze waarop NoV zich verspreidt binnen besmette afdelingen. Beter begrip van de transmissie is cruciaal om beleidsmakers te informeren over optimale preventie- en interventiestrategieën, met name in instellingen met een hoog risico. Binnen deze kaders, was het een specifiek doel om criteria te ontwikkelen voor het gebruik van moleculaire diagnostiek en typering bij bronopsporing. Hiervoor is bij een aantal uitbraken gedetailleerd onderzoek gedaan naar de verspreiding van de infectie, de bijdrage aan de verspreiding van personen zonder ziekteklachten, en factoren die zouden kunnen bijdragen aan vroegtijdige signalering.

Omdat infecties met NoV heel vaak gemist worden, is in eerste instantie de onderdiagnose van NoV in een groot academisch ziekenhuis geëvalueerd, zoals beschreven in **hoofdstuk 2**. Door systematische screening van fecesmonsters van alle personen waarvoor fecesonderzoek werd aangevraagd (in plaats van alleen de aangevraagde test) gedurende een periode van 6 maanden, werd het aantal NoV-diagnoses verdubbeld. Cases die gemist waren in de routinediagnostiek bleken hun oorsprong te hebben in 5 van de 6 NoV-uitbraken die in deze periode plaatsvonden binnen de onderzochte instelling. Niet onderkende NoV-infecties verschilden klinisch niet van NoV-infecties waarbij de diagnose wel gesteld was, en waren kostenverhogend door duurdere diagnostiek en verdergaande transmissie tijdens uitbraken.

Om de verspreiding van het virus binnen deze instellingen (nosocomiale transmissie) verder te onderzoeken, is in **hoofdstuk 3** een (andere) retrospectieve studie opgezet

waarbij nosocomiale transmissie werd geëvalueerd bij patiënten met de diagnose NoV, die waren opgenomen in het ziekenhuis tussen 2002-2007. Bij ca. 60% van de opgenomen patiënten werd de diagnose NoV 5 dagen na opname gesteld, wat suggereert dat de bron van de infectie waarschijnlijk in het ziekenhuis lag. Bestudering van de verzamelde moleculaire virusdata leidde tot nieuwe inzichten, zoals waargenomen in 5 ziekenhuisclusters (uitbraken), waarbij sommige patiënten alleen de polikliniek hadden bezocht, waar ook het virusmonster werd afgenomen. Deze cases waren dus niet opgenomen in het ziekenhuis, wat erop wijst dat deze patiënten bronnen van introductie van het virus kunnen zijn.

De vergaarde inzichten zijn gebruikt in **hoofdstuk 4** om de mate van verspreiding in het ziekenhuis en de bijdrage van verschillende genotypen van NoV in beeld te brengen. Uit zorgvuldig prospectief onderzoek bleek dat NoV-infectie bijna tweemaal zo veel voorkwam dan routinematig herkend, met name onder personeelsleden (met en zonder klachten), en onder patiënten zonder ziekteklachten (buikgriep: braken, diarree), hoewel de uitbraken in het algemeen snel onder controle waren. Uit analyse van moleculaire typeringen, in combinatie met epidemiologische gegevens, bleek dat personeelsleden en patiënten met ziekteklachten het meeste bijdragen aan verspreiding, terwijl NoV-positieve personeelsleden zonder ziekteklachten nauwelijks bijdragen aan virusverspreiding. Dit is opmerkelijk, aangezien bij klachtenvrije infecties grote aantallen virussen kunnen worden uitgescheiden, gedurende enkele maanden na infectie. Afwezigheid van verspreiding door klachtenvrije personeelsleden is belangrijk, vooral in verband met maatregelen om de verspreiding van het virus tegen te gaan. Na verdwijnen van de symptomen van acute ziekte kan zorgpersoneel veilig weer aan het werk, mits men de regels voor hygiëne (handen wassen) zorgvuldig in acht neemt.

Geïnfecteerden besmetten anderen doordat ze het virus uitscheiden. Over NoV-uitscheiding in relatie tot transmissie is weinig bekend, vooral als het gaat om verspreiding tussen de verschillende soorten patiënten en personeelsleden in een gezondheidsinstelling. In **hoofdstuk 5** is de uitscheiding van NoV bij patiënten en medewerkers bestudeerd, door middel van een kwantitatieve PCR-methode. Door middel van een wiskundig model is bepaald hoe de uitscheiding van NoV verloopt: na infectie een snelle stijging, het bereiken van een maximum, gevolgd door een langzame afname. Symptomatisch en asymptomatisch geïnfecteerden bleken eenzelfde uitscheidingspatroon te vertonen. Bovendien werd waargenomen dat zowel

symptomatisch als asymptomatisch geïnfecteerden soms maandenlang NoV kunnen uitscheiden. Ondanks deze langdurige uitscheiding zijn er overigens geen vervolguitbraken gezien die door eenzelfde virusstam werden veroorzaakt.

In alle geobserveerde uitbraken zijn sequenties van het P2-domein als moleculaire marker gebruikt om clusters te classificeren; infecties binnen eenzelfde cluster hebben identieke P2-sequenties. Een validatie van deze methode is nog niet eerder gerapporteerd. Daarom zijn in **hoofdstuk 6** alle vergaarde P2-domeinen van alle uitbraken in detail geanalyseerd. Hierbij werd een pseudo-uitbraak ontmaskerd: een cluster dat geclassificeerd was als 1 uitbraak op basis van epidemiologische gegevens bleek uiteen te vallen in twee onafhankelijke delen. Binnen de onderzochte uitbraken is gebleken dat de P2-marker genoeg resolutie biedt om uitbraken en virusstammen van elkaar te scheiden, en zodoende transmissieketens en bronnen op te sporen. Sequentieveranderingen binnen het P2-domein worden wel gezien, zelfs binnen een enkele uitbraak veroorzaakt door dezelfde stam. Bij een gedetailleerde analyse bleek dat kleine veranderingen (enkele baseparen) voorkomen over het gehele P2-domein en dat zij soms tot aminozuurveranderingen hebben geleid. Toenemende kans op verandering werd gezien vanaf dag 5 na infectie, waarbij na drie weken gemiddeld 10% kans bestaat voor nucleotideverandering; de kans op verandering neemt toe met het verloop van tijd. Hieruit blijkt dat het P2-domein een nuttige marker is om te gebruiken tijdens uitbraken.

Om het begin van uitbraken (introductie van het virus) in kaart te brengen, hebben we uitbraken gevolgd van 2009 tot 2011, waarbij aanvullende gegevens werden verzameld door middel van vragenlijsten, zoals beschreven in **hoofdstuk 7.** Het bleek lastig om zulke gegevens te verzamelen bij patiënten en medewerkers tijdens een uitbraak. Dit onderzoek toont aan dat de uitbraken sterk verschilden in grootte en duur, terwijl zowel patiënten en medewerkers als mogelijke indexcases (eerste infectieuze persoon) konden worden geclassificeerd. Incidenteel bleek het wel mogelijk om een waarschijnlijke introductiegebeurtenis te achterhalen, maar meestal was eenduidige identificatie niet mogelijk. Om virusintroductie beter te kunnen beschrijven zijn meer gegevens en vooral snellere monstername nodig.

In hoofdstuk 8 is uitscheiding van NoV beschreven voor drie chronische uitscheiders, tijdens ziekenhuisuitbraken. Door de moleculaire marker (P2-domein) te gebruiken in combinatie met patiëntgegevens kon worden aangetoond dat transmissie was opgetreden vanuit deze chronische uitscheiders naar andere opgenomen patiënten

binnen het ziekenhuis. Hieruit blijkt dat deze patiënten potentiële bronnen zijn voor NoV-verspreiding binnen het ziekenhuis.
Uiteindelijk zijn **in hoofdstuk 9** alle verzamelde gegevens samengevat en bediscussieerd vanuit de vraag hoe NoV-uitbraken beter kunnen worden gekarakteriseerd, en vooral voorkomen.

Conclusie en aanbeveling

De opsporing van NoV-infecties in zorginstellingen is niet optimaal, wat kan leiden tot verhoogde diagnostiek- en behandelkosten, en complicaties bij kwetsbare patiënten met verstoorde immuniteit. Screening van patiënten bij opname (voornamelijk tijdens het winterseizoen), gebruik van snelle typering, en het (geanonimiseerd) koppelen van gegevens over ziekmeldingen bij personeelsleden en patiënten kunnen bijdragen aan vroegtijdige detectie van clusters.

Curriculum Vitae

Faizel Sukhrie werd op 19 maart 1984 geboren in Nickerie, een kleine stad in Suriname die grenst aan Brits-Guyana en Brazilië. In 2004 behaalde hij zijn diploma als Medisch Analist in diagnostisch onderzoek aan het Natuur Technisch Instituut (NATIN) in Paramaribo. Tijdens deze studie liep hij stage binnen verschillende ziekenhuislaboratoria in Suriname.

Aansluitend begon hij met de opleiding Medische Microbiologie en Moleculaire Biologie aan de Hogeschool Leiden. Tijdens deze studie liep hij stage bij Crucell Holland B.V., op de afdeling quality control. Hier voerde hij ook zijn afstudeerproject uit, met als onderwerp de implementatie en validatie van biologische indicatoren binnen de nieuwe pilot plant van Crucell, onder begeleiding van Drs. Annelies van Goor, Ing. Arjan Roozen en Ing. Denny Stouten.

Na het behalen van zijn Bachelor of Applied Science, begon hij met de masteropleiding Biomedische Wetenschappen (Biomedical Sciences) aan de Vrije Universiteit Amsterdam met twee verschillende specialismen: International Public Health en Infectious Diseases. Hiervoor deed hij een eerste afstudeeronderzoek bij de GGD in Den Haag, met als onderwerp de associatie van diabetes type 2 met vitamine D-deficiëntie bij Aziatische vrouwen, onder begeleiding van Drs. Trudy van Ommeren en Drs. Balram Bissumbar. De tweede stage liep hij bij het VU Medisch Centrum op de afdeling Medische Microbiologie onder begeleiding van Dr. Jeroen Geurtsen en Dr. Ben Appelmelk.

Het onderwerp hier was een knock-out *Mycobacterium tuberculosis* te construeren met een alpha-glucan deficiëntie, die zou kunnen bijdragen aan de ontwikkeling van een vaccin tegen tuberculose.

Na het behalen van het masterdiploma is hij begonnen met zijn promotieonderzoek op de afdeling Virologie van de Erasmus Universiteit en het Erasmus Medisch Centrum in Rotterdam, in samenwerking met de afdeling Virologie van het Centrum voor Infectieziekten Bestrijding (CIB) aan het Rijksinstituut voor Volksgezondheid en Milieu in Bilthoven, onder begeleiding van Prof. Dr. Marion M.P.G. Koopmans, Prof. Dr. Peter F.M. Teunis en Dr. Harry Vennema.

Publicaties

1. Beersma MF, Sukhrie FH, Bogerman J, Verhoef L, Mde Melo M, Vonk AG, Koopmans M: **Unrecognized norovirus infections in health care institutions and their clinical impact**. *J Clin Microbiol* 2012, **50**(9):3040-3045.
2. Sukhrie FH, Beersma MF, Wong A, van der Veer B, Vennema H, Bogerman J, Koopmans M: **Using molecular epidemiology to trace transmission of nosocomial norovirus infection**. *J Clin Microbiol* 2011, **49**(2):602-606.
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4. Sukhrie FH, Teunis P, Vennema H, Bogerman J, van Marm S, Beersma MF, Koopmans M: **P2 domain profiles and shedding dynamics in prospectively monitored norovirus outbreaks**. *J Clin Virol* 2013, **56**(4):286-292.
5. Sukhrie FH, Teunis P, Vennema H, Copra C, Thijs Beersma MF, Bogerman J, Koopmans M: **Nosocomial transmission of norovirus is mainly caused by symptomatic cases**. *Clin Infect Dis* 2012, **54**(7):931-937.
6. Teunis P, Heijne JC, Sukhrie F, van Eijkeren J, Koopmans M, Kretzschmar M: **Infectious disease transmission as a forensic problem: who infected whom?** *Journal of the Royal Society, Interface / the Royal Society* 2013, **10**(81):20120955.

Erasmus MC
Universitair Medisch Centrum Rotterdam

PhD Portfolio Summary

Summary of PhD training and teaching activities

Name PhD student: Faizel Haiaat Alie Sukhrie	PhD period: September 2008 – February 2013
Erasmus MC Department: Virology	Promotors: Prof. dr. M.P.G. Koopmans, Prof. dr. Peter F.M. Teunis
Research School: Molecular Medicine Postgraduate School	Co-promotor: Dr. Harry Vennema

1. PhD training

	Year	Workload (Hours)
General academic skills		
- Oral presentations: design and performance (RIVM)	2008	20
- Design of scientific posters (RIVM)	2008	8
- Biomedical English Writing and Communication (RIVM)	2009	40
- Biomedical English Writing and Communication (ErasmusMC)	2010	40
Research skills		
- Statistics (data analysis training with scripts in R), RIVM, Bilthoven	2012	16
- AIO meetings (paper discussions) RIVM, Bilthoven	2011-2013	32
In-depth courses (e.g. Research school, Medical Training)		
- Virology course (Erasmus MC)	2010	40
- NoV extraction protocol VWA Zutphen	2010	8
- PhD session EMC (management, PhD at ErasmusMC	2010	8
- Molecular analysis (Rotterdam)	2011	16
- Analysis of serological data (statistical analysis)	2012	8
Presentations (oral/ poster)		
- PhD Project Proposal (LIS VIR) RIVM, Bilthoven (oral)	2008	8
- WHO meeting (RIVM, Bilthoven), (oral)	2009	8
- PhD retraite on beach session (Zandvoort), (oral)	2009	8
- LIS audit (poster) RIVM, Bilthoven	2010	8
- European Society for Clinical Virology, Como, Italy (poster)	2010	8
- Presentation 4th international congress of *caliciviruses*, Chile, Santa Cruz (oral)	2010	8
- Presentation c*alicivirus* meeting in Madeira, Portugal, (oral)	2011	8
- Presentation research outcome at the Epidemiology department, RIVM, the Netherlands	2011	8
- Presentations LIS VIR meetings RIVM, Bilthoven (4 x), (oral)	2008-2013	32

- Final presentations PhD project LIS/ EPI meetings	2013	32
(Inter)national conferences		
- DAVS (Dutch Annual Virology Symposium)	2009	16
- European Society for Clinical Virology, Como, Italy	2010	40
- 4th International Congres of Caliciviruses, Santa Cruz, Chile	2010	40
- Annual meeting European Society for Clinical Virology, Funchal, Madeira.	2011	40
- Spring meeting NVMM, Papendal, the Netherlands	2011	32
- Norovirus winter meeting, Lubeck, Germany.	2012	40
Seminars and workshops		
- Training Poster Presentation (RIVM), Bilthoven	2008	8
- Bionumerics training (RIVM, Bilthoven)	2009	8
- Scientific writing English (linguarama), (RIVM)	2009	40
- Proneri (RIVM) Bilthoven (PhD thesis and publishing articles)	2009	8
- Molecular Medical Microbiology, RIVM, Bilthoven	2010	8
- Phylogeny course RIVM, Bilhoven	2011	16
- FES Avian Influenza (end presentation)	2012	4
- Statistics course (RIVM Bilthoven)	2012	8
- Communication and media training	2013	8
- Workshops Proneri (PhD network RVM)	2008-2013	24
- Career options after having finished your PhD	2008	
- How to get it Published	2009	
- Software and management Skills	2010	
Didactic skills		
- Training norovirus extraction protocol (VWA Zutphen)	2010	8
2. Teaching activities		
	Year	**Workload (Hours)**
- Supervision BSc. graduate student (trainee for 7 months)	2011	160
Lecturing		
- Final Year students (5 months)	2011	100
Supervising practicals and excursions		
- Lab training final year students	2011	100
- Lab training en sequence analysis PhD students	2012	40
Supervising Master's theses		
- Student from Hogeschool Utrecht	2011	80