NOROVIRUS GENETIC DIVERSITY

FROM WITHIN PATIENT VIRAL EVOLUTION TO GLOBAL DISTRIBUTION

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Norovirus Genetic Diversity – from within patient viral evolution to global distribution

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Genetische diversiteit van norovirus –
van virale evolutie binnen patiënten tot wereldwijde verspreiding

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The majority of all non-bacterial gastroenteritis outbreaks are caused by human noroviruses and norovirus infection is associated with 18% of all cases of gastroenteritis worldwide[1, 2]. These viruses are highly infectious, as even a few particles can cause disease, and infected individuals shed high loads of virus[3, 4]. Transmission occurs by the faecal–oral route, either through contact with infected individuals or through exposure to contaminated food and water or to infectious aerosols that are produced by vomiting[5-8]. As a result of this high infectivity and efficient transmission, newly emerged strains of norovirus can cause global epidemics[9]. Norovirus infections are self-limiting in healthy individuals but are associated with severe complications in immunocom-promised individuals, the elderly and young children[10-14].

Classification

The genus *Norovirus* belongs to the family of *Caliciviridae* and four other genera in this family are recognised by the International Committee on Taxonomy of Viruses (ICTV), i.e. *Sapovirus*, *Vesivirus*, *Lagovirus* and *Nebovirus*. The genera *Vesivirus* and *Lagovirus* contain some important veterinary pathogens such as feline calicivirus (vesivirus) and rabbit hemorrhagic disease virus (lagovirus)[15, 16]. With the exception of an anecdotal zoonotic vesivirus infection, only members of the noroviruses and sapoviruses have been found to infect humans. Sapoviruses mainly cause mild gastroenteritis in children up to 5 years of age, while norovirus can infect humans in all age groups[2, 17]. The *Norovirus* genus is divided in seven genogroups, which are further subdivided into approximately 40 genotypes (figure 1)[18].

Figure 1 ORF1 and ORF2 phylogenies. Two regions of the norovirus genome are used to classify strains of norovirus: the region of ORF1 that encodes the RNA-dependent RNA polymerase (RdRp), and ORF2, which encodes the structural capsid protein VP1. Genetic diversity and frequent recombination events between ORF1 and ORF2 have resulted in phylogenetic topologies that, although similar, are not identical, as shown in unrooted maximum likelihood trees estimated for ORF1 (part **a**) and ORF2 (part **b**) sequences of all norovirus ORF1 and ORF2 genotypes[18]. Owing to the frequent occurrence of recombination events between ORF1 and ORF2 sequences, a dual nomenclature for norovirus classification using both sequences encoding RdRp and sequences encoding VP1 has been proposed[120]. Note that the nomenclature of genogroups GIV, GVI and GVII has not been consistent: genogroups GIV and GVI were initially classified as a single genogroup, which was known as genogroup GIV, and norovirus strains in genogroup GVII have also been classified in the past as belonging to genogroup GVI. However, we have chosen to use seven genogroups, as proposed by Vinjé[18]; this reclassification is based on amino acid divergence. The scale bar reflects the number of nucleotide substitutions per site. Part b is modified from *J. Clin. Microbiol.*, 2015, 53, 373–381, http://dx.doi.org/10.1128/JCM.01535-14 and amended with permission from American Society for Microbiology.

a ORF1 (RdRp)

0.4

b ORF2 (VP1)

0.5
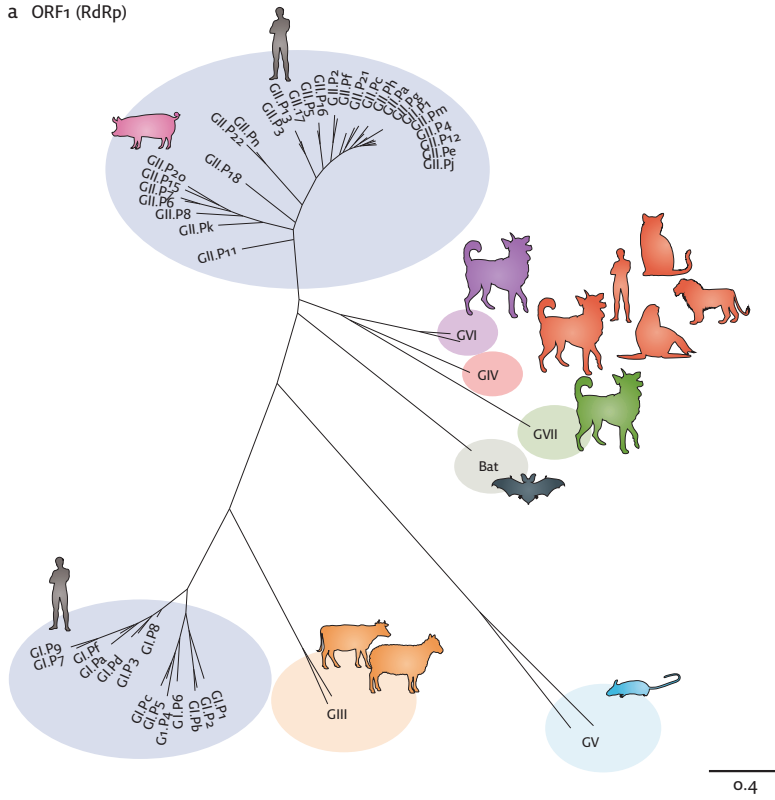
1
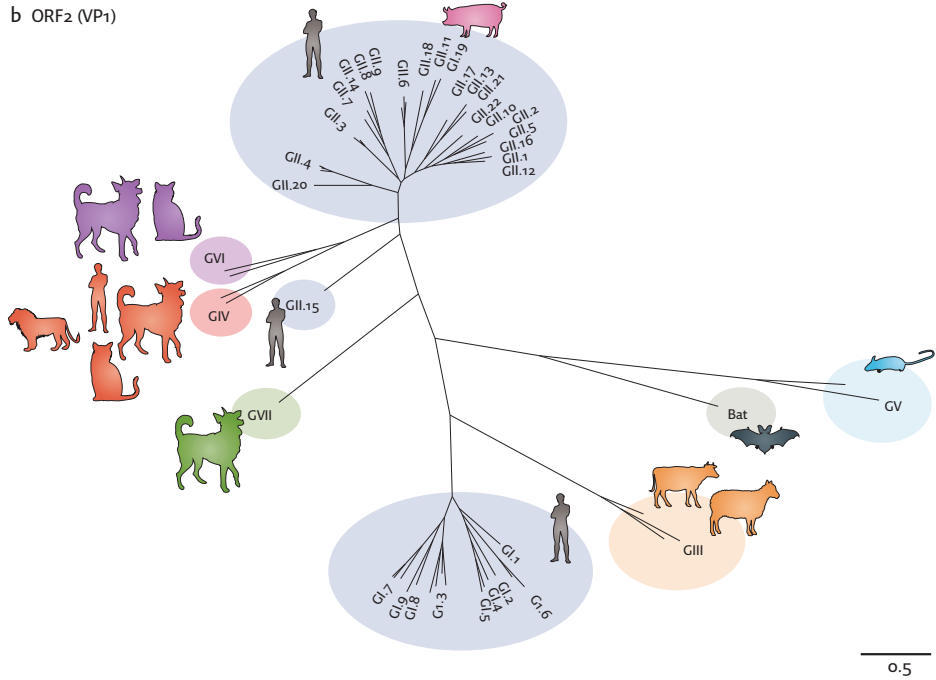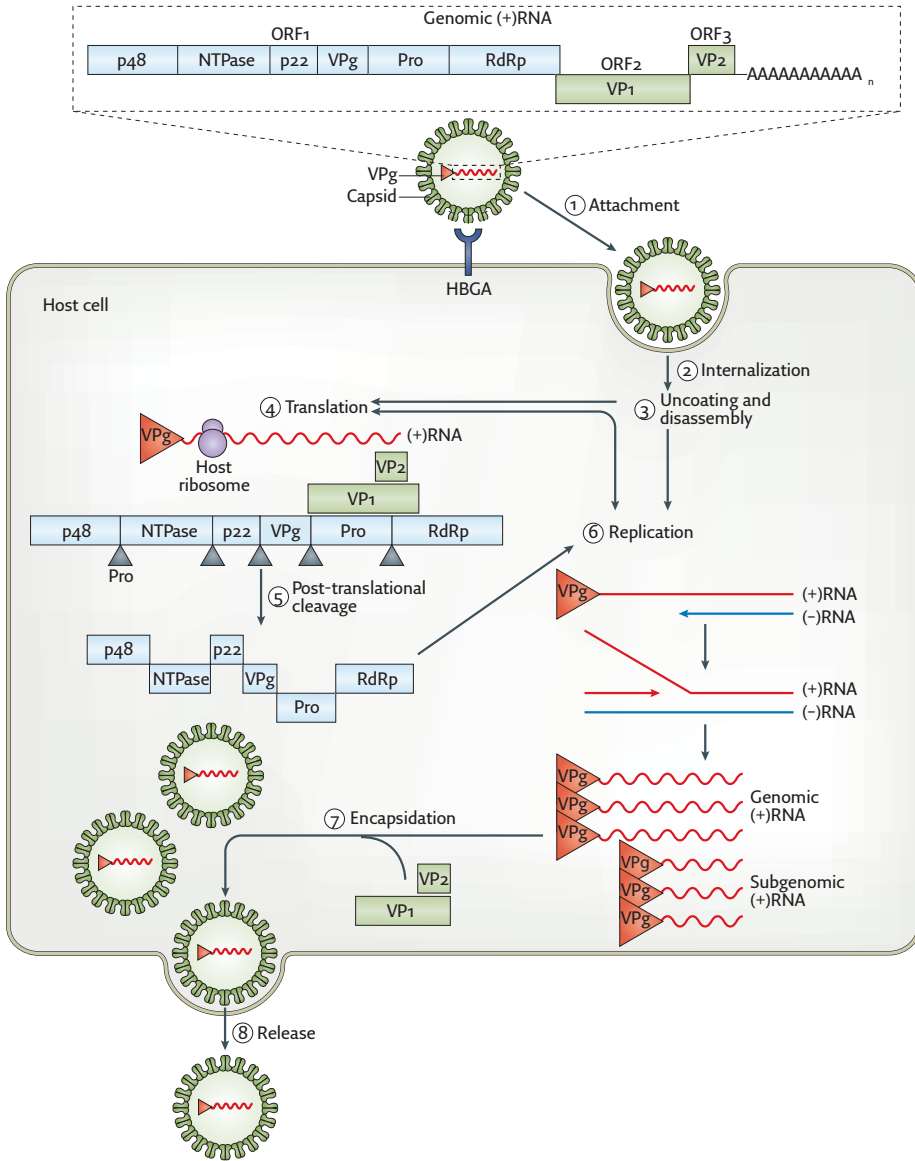
Figure 2 The composition and life cycle of human noroviruses. The norovirus genome has three ORFs, which encode a polyprotein — encompassing six individual non-structural proteins — and the structural proteins VP1 and VP2. The genome, in the form of a positive-sense RNA strand ((+) RNA), is encapsulated in a capsid that is formed by VP1 and VP2. The capsid attaches to the cell surface through interactions between VP1 and host histo-blood group antigens (HBGAs) (step 1), and is subsequently internalized, uncoated and disassembled (steps 2,3). The (+)RNA is then transcribed and translated in the cytoplasm of the host cell. Translation is mediated by host trans-

lation factors that are recruited by the non-structural virus protein VPg, which covalently binds to the 5'end of the genome (step 4). The polyprotein that is encoded by ORF1 is post-translationally cleaved (step 5) by the virus-encoded protease, Pro (also known as NS6 or 3Clike), into individual proteins: p48 (also known as NS1/2 or Nterm), NTPase (also known as NS3 or 2Clike), p22 (also known as NS4 or 3Alike), VPg, Pro and RNA-dependent RNA polymerase (RdRp). During genome replication, (+)RNA is transcribed into negative-sense RNAs ((−)RNAs), which are used as templates for the synthesis of new genomic and subgenomic (+)RNAs, respectively (step 6). Subgenomic (+)RNAs contain only ORF2 and ORF3, and are used for the production of VP1 and VP2. During encapsidation (step 7), genomic — and possibly subgenomic — (+)RNAs are packaged into new virions, which are subsequently released from the infected host cell (step 8), although the mechanism by which release occurs remains largely unknown.

Figure 3 X-ray structure of (a) the Norwalk virus capsid and (b) capsid subunit structure (figure 3 was kindly provided by B.V.V. Prasad, Baylor College of Medicine, Houston, TX, USA). NTA = N-terminal arm; P1 and P2 = P1 and P2 subdomains; S = S domain.

Genome organisation

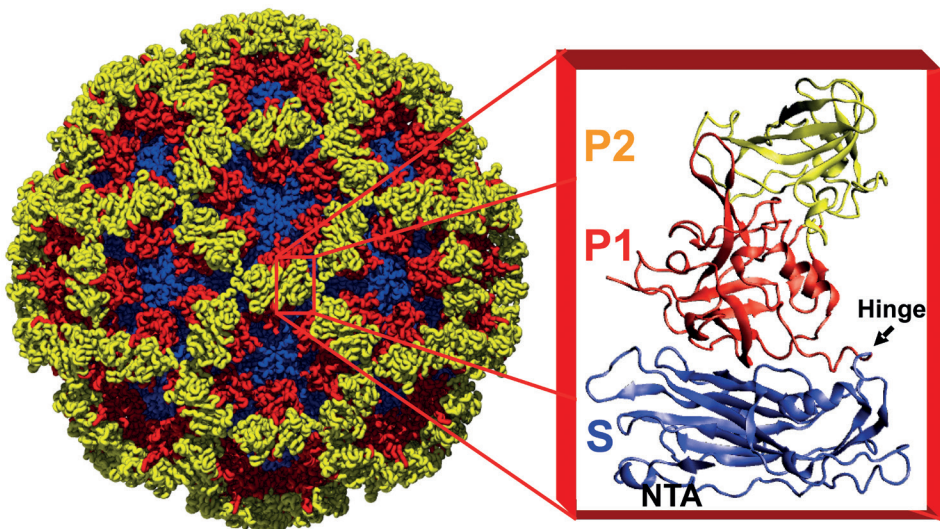Noroviruses have a positive linear single-stranded RNA genome with a size of approximately 7.5 kb that is organised in three open reading frames (ORF's) (figure 2). ORF1 encodes for a polyprotein containing all seven non-structural proteins that is produced as one large polyprotein and then cleaved into individual proteins[19]. The non-structural proteins are essential for the production of new viruses in infected cells but do not form part of virus particles. ORF2 encodes the major structural protein VP1 (alternative name: capsid protein) and ORF3 encodes the VP2 protein (or minor capsid protein). The VP2 protein is assumed to be a minor structural protein since each norovirus virion only contains one or two copies[20]. During replication of the RNA genome, the RNA-dependent RNA polymerase does not have a complementary strain for proofreading activity. Therefore, noroviruses, like other RNA viruses, have a high mutation rate of 1-4×10^{-3} substitutions per nucleotide per year, while DNA-dependent DNA polymerases (DNA viruses or cellular organisms) have a mutation rate of 1×10^{-6} - 10^{-8} substitutions per nucleotide per year[21, 22]. Hence, RNA viruses are highly diverse and have a much faster evolution rate compared to their host[23]. Due to this high mutation rate, a norovirus population in a single host exists of a diverse mixture of nearly identical strains (quasi-species). This property, common to several RNA viruses, calls for the genetic flexibility of these viruses.

Virus characteristics

Noroviruses do not have a lipid envelope, but the genetic material is protected by a capsid of VP1 proteins. With this capsid noroviruses can survive the acidic environment of the stomach and persist for a long period in the environment, although the particles appear to be less stable at elevated pH[4]. The VP1 protein is the major structural protein of norovirus and each virion contains 180 copies (90 dimers) of the VP1 protein symmetrically arranged (figure 3). The virus attaches with the VP1 protein to the host cell receptor and VP1 therefore plays an important role in virus-host interactions[24]. Cryo-electron microscopy studies have shown that the VP1 protein can be divided in two pieces: the shell domain and the protruding domain (S and P domain) (figure 3). All S-domains of the 180 VP1 copies form a shell around the viral RNA (icosaheder) and the P domains form arch-like structures surrounding the shell. The P domain is connected to the S-domain *via* a flexible hinge and can be further subdivided in the P1 and P2 domain of which P2 mostly protrudes[25].

Virus shedding and infectivity

Noroviruses enter the body via the mouth and virions pass the acidic environment of the stomach. Replication takes place in the upper intestinal

tract, but the exact cell type is unknown[26]. Virions are shed in high quantities (10^7-10^{10} RNA copies per gram) in faeces[27-30] and have been detected with lower loads in vomitus as well[31]. Infected individuals shed virus in highest quantities during the acute phase and shedding continues during an asymptomatic phase which can last 9-56 days[3, 27]. Prolonged and asymptomatic shedding has been reported for children (up to 100 days) and immunocompromised patients can suffer from prolonged illness and shedding, which can last up to several years[14, 29, 32-35].

Norovirus is highly infectious with an estimated basic reproduction number (R_o) of more than 14 during an outbreak in a scouting camp[36], which means that during the infection period each case, on average, has infected 14 other cases. The implementation of hygiene measures on the scouting camp had a large effect on the R_o, which decreased to 2[36]. This high R^o is in part explained by the low dose required for infection. Therefore, even removal of several logs of virus during contamination events may still not be enough to stop spread of disease[37].

Incubation period and symptoms

In healthy individuals, norovirus causes mild gastroenteritis and symptoms are generally self-limiting. From outbreak studies it is known that symptoms usually last < 1-5 days and include vomiting, non-bloody diarrhoea, abdominal cramps and pain, nausea, and fever[38-40]. In children, vomiting is more common whereas adults endure more often diarrhoea[40]. Norovirus outbreaks tend to have a longer incubation period compared to bacterial gastroenteritis outbreaks. In 85% of norovirus outbreaks, the incubation period was > 24 h compared to 39% for outbreaks caused by a bacterial agent[41]. In 1982, Kaplan *et al.* reviewed 642 acute gastroenteritis outbreaks to extract criteria for identification of Norwalk-like associated outbreaks[42]. These Kaplan's criteria are 1) > 50% of cases vomits, 2) mean or median incubation period of 24-48 hours post infection, 3) duration of illness between 12 and 60 h, and 4) absence of etiological bacteria in stool samples. In 2006, these criteria were re-assessed and revealed to be highly specific (99%), moderately sensitive (68%) and a useful diagnostic tool to distinguish norovirus outbreaks from bacterial outbreaks in outbreak situations where other diagnostic methods are not yet available[43].

Transmission

Surveillance through national and international collaborative networks, such as CaliciNet and NoroNet, has provided important insights into how different strains of human norovirus correspond to modes of transmission and outbreak settings. Strains of the GII.4 genotype caused 70–80% of all reported outbreaks

over the past 13 years or so[44], but the prevalence of infecting genotypes differs between human populations and routes of transmission[45]. Genotype GII.4 is more often associated with transmission mediated by person-to-person contact than with other types of transmission, whereas non-GII.4 genotypes, such as GI.3, GI.6, GI.7, GII.3, GII.6 and GII.12, are more often associated with foodborne transmission[6]. GI strains are more often associated with waterborne transmission than GII strains[8], a trait that may relate to the proposal that GI strains have a higher stability in water than GII strains. As strains may adapt to host factors that vary according to the population that is infected, such as age, health and pre-existing immunity, differences in the epidemiology of norovirus genotypes in community settings are likely to influence the evolution of the genotypes.

Foodborne transmission is an important route for the global spread of noroviruses[6] and can occur either when food handlers contaminate food on site or during the earlier steps of food production[46]. For example, shellfish that are cultivated in coastal areas can be contaminated by faecal discharge[47], and products such as fresh and frozen berries can be contaminated by irrigation with sewage-contaminated water or by contact with infected personnel during harvesting and processing. Foodborne outbreak events occur frequently and are a potential source of transmission of strains between different parts of the world, given the globalization of the food chain. These outbreaks can include mixtures of norovirus strains[8], thus increasing the risk of viral recombination. The global scale of foodborne outbreaks of noroviruses can be difficult to recognize because the epidemiology of outbreaks is often tracked independently by individual countries; nonetheless, retrospective studies have shown that approximately 7% of foodborne outbreaks of noroviruses are part of an international event with a common source[48]. Globally, noroviruses rank among the top causes of foodborne disease[49].

Nosocomial transmission of noroviruses in hospitals is a major burden for inpatient services[2]. Individuals may shed norovirus particles in considerable numbers for several weeks after the resolution of symptoms[39], possibly acting as a source for nosocomial transmission[50]. However, analyses of nosocomial outbreaks suggest that most of these outbreaks are the result of transmission from symptomatic shedders[50]. In a hospital setting, immunocompromised patients who are chronically infected with norovirus and are symptomatic can act as a reservoir of the virus and may contribute to nosocomial transmission[51, 52]. As a consequence of prolonged shedding and limited immune pressure, these immunocompromised patients can harbour numerous norovirus variants. The

intrahost viral variation in a chronic shedder can mimic the antigenic variations that are seen between consecutive human norovirus pandemics, and some of these variants may be able to escape herd immunity[53].

Infections of humans with animal norovirus strains have not yet been reported, but there is some evidence for the transmission of noroviruses between different host species. Human noroviruses have been detected in the stool of pigs, cattle and dogs[54, 55], and gnotobiotic calves and pigs can become experimentally infected with human GII.4 strains[56, 57]. Furthermore, canine seroprevalence to different human norovirus genotypes resembles the seroprevalence in the human population[58], and serum antibodies against bovine and canine noroviruses have been detected in humans, with higher levels in veterinarians than in the general population[59, 60].

Virus detection

Norovirus was discovered in 1972 by Electron Microscope (EM) analysis of stool samples from an outbreak of acute non-bacterial gastroenteritis with unknown aetiology at an elementary school in Norwalk, Ohio[61]. EM can visualize norovirus particles, but because the concentration of particles required for reliable detection by EM is estimated to be around 10^5 or higher, it is relatively insensitive compared to molecular methods. The time of sampling is critical for a successful diagnosis with EM. In 11 of 23 norwalk challenged volunteers, virions could be detected in at least one specimen within 72 hours after onset of disease [62]. Before the onset of disease all specimens were negative and after 72 hours post challenge only 2 of 11 specimens were positive.

Reverse transcriptase polymerase chain reaction (RT-PCR) is nowadays the most frequently used technique for detection of norovirus RNA in stool samples. RT-PCR uses a reverse transcriptase enzyme to reverse transcribe RNA into cDNA (complementary DNA). The cDNA molecules are subsequently amplified and quantified using a fluorescent dye. Various RT-PCR assays are developed for norovirus detection in clinical samples like faeces and vomitus, in food samples, and environmental samples. Due to the high sequence variability among norovirus strains, most RT-PCR assays use primers that target a conserved region in ORF1 (coding for the viral RNA polymerase) or a conserved region in the ORF1-ORF2 junction region[63, 64]. Although sensitivity of these assays for detection of viruses from different genotypes may differ, this problem seems to have been overcome in more recent PCR protocols[65]. Nevertheless, the potential differences in sensitivity of diagnostic assays should always be considered in gastroenteritis outbreak situations that fulfil Kaplan's criteria,

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but without positive PCR results. Of note: this may be particularly the case in foodborne disease outbreaks, where less common genotypes are seen[66]. This also explains why genotyping may be important in outbreak investigations. Immunoassay (EIA) like i.e. IDEIA Norovirus (Thermo Fisher Scientific, Basingstoke, United Kingdom), RIDASCREEN Norovirus (R-Biopharm, Darmstadt, Germany) and SRSV(II)-AD (Denka Seiken Co. Ltd., Tokyo, Japan) are available to detect norovirus antigen in stool samples. The advantage of EIA assays compared to RT-PCR based methods is the simplicity and rapidity of the assay. No specialised equipment is required and results can be ready within four hours. These three assays make use of a sandwich ELISA format. The SRSV(II)-AD and IDEIA assays use monoclonal antibodies against GI and GII to capture the norovirus antigens in stool samples[67, 68]. Horseradish conjugated rabbit polyclonal antibodies directed against a pool of GI and GII VLP's are subsequently added to detect the antigens. Not much is known about the reagents used for the RIDASCREEN assay, but it has been described that it does make use of monoclonal antibodies for antigen capture and a secondary antibody for antigen detection[69]. Comparative studies have tested sets of stool samples with the commercial ELISA assays and showed a wide range of sensitivity and specificity values. Among these studies the median sensitivity was 56% (range: 31-92) and median specificity of 95% (range: 47-100%) depending on the type of assay and strains tested[67, 68, 70-75]. This low sensitivity precludes use of these assays for individual patients, but diagnosis of outbreaks may be possible[76]. Nevertheless, due to the high genetic and antigenic diversity of norovirus strains, certain genotypes can be missed with these assays and therefore they should preferably be used in combination with a confirmation of negative samples by RT-PCR.

Host immune response

Why norovirus infections can result in severe complications and chronic infections in certain high-risk groups is not fully understood, as the factors that offer protection to the host during infection with human noroviruses are not fully known. For murine noroviruses, components of the adaptive immune system, including B cells, CD4+ T cells and CD8+ T cells, are required for efficient viral clearance from the intestine and intestinal lymph nodes[77]. In addition to the adaptive immune system, the innate immune system seems to have an important role in the clearance of infection.

Early studies of infection in humans suggested that the acquisition of protective immunity to noroviruses is short term[78], but more recent reports indicate that protective immunity is longer lasting than initially thought[79, 80]. Owing to the

historical lack of a cell culture system for the study of norovirus replication, virus neutralization has not been measured directly and the measurement of the inhibition of VLP binding to HBGAs has instead been used as a surrogate assay [81]. In challenge studies in humans and chimpanzees, increased serum titres of antibodies that inhibited VLP binding to HBGAs correlated with a reduction in the rate of infection and in disease severity[82, 83]. In human challenge studies, an early mucosal immunoglobulin A (IgA) response was associated with protection against norovirus infection[84]. Furthermore, pre-existing norovirus-specific IgA in saliva and norovirus-specific memory IgG cells were associated with protection from gastroenteritis[85]. Moreover, pre-existing faecal norovirus-specific IgA was associated with a reduction in peak viral load, and the magnitude of faecal levels of IgA measured one week after infection correlated with a shorter duration of shedding[85]. In conclusion, these findings support a role for host immune responses in reducing the viral load, the duration of virus shedding and the severity of disease.

Attachment to histo-blood group antigen variants

HBGAs are glycans that are expressed on the surface of specific cells — and present in saliva and other bodily secretions — and are determinants of both the ABO blood group and Lewis blood group systems[86]. In certain cell types, α(1,2)-fucosyltransferase 2 (FUT2; also known as galactoside 2αlfucosyltransferase 2) adds a fucose group to precursors of HBGAs, generating H HBGAs, and subsequent reactions generate A and B HBGAs. The binding specificity of norovirus VP1 to different HBGAs differs among norovirus genotypes and genogroups[87], resulting in differences in the susceptibility of human individuals to specific strains of norovirus[84, 88]. Individuals who lack FUT2 are known as non-secretors, as A, B and H HBGAs are not present in the bodily secretions of these individuals[89]. Around 20% of Northern Europeans are non-secretors[88], and children of Mesoamerican ancestry are more likely to be secretors than children of European or African ancestry[90]. Non-secretors have been shown to be less susceptible to infection with several GI and GII strains of norovirus[84, 88, 91]. Differences in the expression of HBGAs have a major effect on the susceptibility of individuals to norovirus infections and on the pathogenesis of norovirus strains, as shown in several studies, including a human challenge study with a norovirus GII.4 Farmington Hills 2002 strain. In healthy adults, challenge resulted in the infection of 70% of those individuals with a functional FUT2; of these, 57% developed symptoms of infection. By contrast, only a single individual (6%) was infected in the group without a functional FUT2, and this individual displayed minimal disease[92]. The HBGA specificities of norovirus genotypes, including GII.4 strains, have been comprehensively reviewed elsewhere[93, 94].

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Genetic diversity and evolution

Viruses in the genus *Norovirus* can be found in a wide range of hosts, such as humans, rodents, felines, canines, sea lions, pigs, sheep, cattle and bats[18, 95, 96] (figure 1). The nucleotide sequences of the genomes of different norovirus genogroups share only 51–56% similarity with one another, and the diversity between genogroups is even higher when comparing only ORF2 sequences rather than whole genomes[87, 97]. Despite frequent recombination and possible differences in selection pressures between ORF1 and ORF2, the phylogeny of ORF1 has a similar topology and a similarly high genetic diversity to the phylogeny of ORF2[18] (figure 1). Intriguingly, some outbreaks are caused by strains of norovirus that are genetically similar or identical to strains that were isolated 10–15 years earlier, which raises questions about the reservoirs in which these viruses are maintained between outbreaks[8]. Surveillance studies have shown that globally circulating GII.4 strains are frequently replaced by newly emerged antigenically divergent GII.4 strains, which indicates that an immunogenic pressure influences the evolution of noroviruses, at least for the GII.4 genotype[87]. Importantly, the emergence of antigenically divergent GII.4 strains coincides with an increase in norovirus outbreak activity[98]. Bioinformatic analyses and *in vitro* assays have shown that GII.4 strains have high rates of mutation and evolution, which probably facilitate the emergence of these antigenically divergent strains[99]. Molecular epidemiology of GII.4 isolates collected across the globe showed that some GII.4 lineages that are able to cause widespread regional epidemics were nevertheless geographically limited[9]. The failure of these epidemics to spread throughout the world could be due to differences in the genetic and microbial makeup of the host or differences in the previous exposure of host populations to noroviruses. Since 1995, six antigenically variant GII.4 strains have resulted in pandemics: US 1995/96, Farmington Hills 2002, Hunter 2004, Den Haag 2006b, New Orleans 2009 and Sydney 2012[100]. The emergence of the Farmington Hills antigenic variant in 2002 coincided with an increase in the number of reported norovirus outbreaks[101], which was confirmed by phylodynamic reconstruction to reflect a true increase in infections rather than reporting bias[98].

Prevention

In health care institutions, outbreak management focuses on preventing further spread of the virus by containment of infected individuals and hygienic measures. Hand washing with antiseptic soap for 10 seconds is the key hygienic measure and has demonstrated to prevent further spread of health-care associated infections (bacteria and viruses)[102]. Evidence for effect of hand disinfectants on infectivity is difficult to obtain, because the human noroviruses

cannot be grown in cell culture and animal noroviruses and caliciviruses have different properties[103]. Hand washing with ethanol based solutions or wipes have shown not to be effective for significantly reducing viral concentration[102]. Noroviruses are very stable on environmental surfaces, like water taps, door-handles or cutting plate, and require chemical disinfection with high concentration of hypochlorite, detergents based on hydrogen peroxide or phenolic-based cleaning solutions[37, 104-106].

Clinical intervention efforts for norovirus infection are hampered by the lack of a licensed vaccine, despite important advances in vaccine development, and limited evidence for the success of the antiviral treatment options that are currently available. Several individuals who were chronic shedders have been successfully treated with oral human immunoglobulin, although in some patients treatment did not result in clearance of the virus[107, 108]. Additional studies will be required to determine whether the route of administration and/or the levels of antibodies that are specific to the infecting strain of norovirus are important factors in the success rate of human immunoglobulin treatment. The ability of immunoglobulin to limit infection was also seen in a mouse model of norovirus infection following intraperitoneal administration of immunoglobulin[109]. Another strategy that may clear norovirus infections in immunocompromised patients is the partial restoration of the immune system, whether by reducing, temporarily discontinuing or changing immunosuppressive drugs[110]. However, this should be done with caution, and is not possible for all patients.

Antivirals, including nitazoxanide, ribavirin and interferons, have been shown to inhibit norovirus replication in cell culture-based replicon systems, mouse models or infected human individuals[111-116]. Oral treatment with nitazoxanide, an agent that has broad antimicrobial activity, resulted in clinical resolution of acute gastroenteritis in a patient who was chronically infected, although asymptomatic shedding was observed for another month[113]. Nitazoxanide also reduced the duration of symptoms in a small randomized, double-blind, placebo-controlled clinical trial[112]. Two chronically infected immunocompromised individuals were successfully treated with oral ribavirin, which is a broad-spectrum antiviral agent, although a similar treatment was unsuccessful in two other patients[114].

Historically, the development of a norovirus vaccine has been hampered by the lack of a small-animal model and a cell culture system, both of which have been described only recently, and licensed vaccines are not yet available[117]. Nevertheless, the first norovirus vaccines have now completed Phase I and

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Phase II clinical trials[117]. These vaccines are based on VLPs of the GI.1 genotype or, in the case of the bivalent vaccine, contain both GI.1-derived VLPs and VLPs based on the consensus sequence of several GII.4 variants[117]. The clinical trials showed an induction of antibody responses that occurred regardless of whether the vaccine was administered intramuscularly, orally or intranasally[118]. In a clinical trial with healthy volunteers, intramuscular vaccination with bivalent VLPs did not significantly reduce the incidence of protocol-defined illness after challenge with a GII.4 strain of norovirus. However, the vaccination was able to reduce the incidence and severity of vomiting and diarrhoea[119].

Scope of the thesis

The aim of this thesis was to get a better understanding of the global norovirus diversity, with a focus on the role of chronic norovirus infection on virus diversity, antigenic variation and evolution. The obtained knowledge can be used to predict severe norovirus outbreak seasons, is useful for hospital hygiene and infection control guideline improvement, and is important for future vaccine development.

We first describe major changes in the global norovirus diversity. In **chapter 2.1** the emergence of the GII.4 Sydney 2012 variant is described and in **chapter 2.2** we describe the emergence of GII.17 and replacement of GII.4 in Asia, and the possible consequences for the global public health community. In **chapter 3** we show an integrated analysis of 10 years molecular and epidemiological norovirus surveillance via the international NoroNet network and describe a future perspective on norovirus surveillance.

Recent changes in the norovirus diversity raises questions on the norovirus prevalence before the introduction of molecular techniques for norovirus detection. Since historical faecal sample collections are exceedingly rare, we developed a multiplex serological assay for the detection of norovirus antibodies in human serum samples and used this assay to study the norovirus seroprevalence in historical and recent serum collections (**chapter 4**).

Chronic norovirus infection is a recently described phenomenon among immunocompromised patients and it has been hypothesized that chronic infection plays a role in the development of new norovirus drift variants. In **chapter 5** we study the prevalence of chronic norovirus infection among solid organ transplant recipients in a tertiary care hospital. In **chapter 6** we investigated the genetic and antigenic changes, and quasi species diversity of the within-host virus population among longitudinal samples of patients with chronic norovirus infection by using next-generation deep sequencing technology.

In **chapter 7** we summarize the findings of this thesis and discuss the results in relation to other (recent) norovirus scientific publications.

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Indications for worldwide increased norovirus activity associated with emergence of a new variant of genotype II.4, late 2012

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2.1

Abstract

Globally, surveillance systems showed an increase in norovirus activity in late 2012. Molecular data shared through the NoroNet network suggest that this increase is related to the emergence of a new norovirus genotype II.4 variant, termed Sydney 2012. Healthcare institutions are advised to be prepared for a severe norovirus season.

In the United Kingdom (UK), the Netherlands, and Japan, norovirus (NoV) epidemiological and laboratory surveillance systems show increased levels of NoV activity compared to previous seasons, in late 2012[1-3]. Similarly, increases have been noted in Australia, France and New Zealand (unpublished data). At this stage, and with the limited surveillance of NoV in most countries, it is difficult to conclude if these increases denote early seasonal activity or truly increased incidence, although for the UK the latter has been suggested. On 29 November, and on 4 and 6 December, ProMed (http://www.promedmail.org/) messages reported a dramatic rise in NoV hospital outbreaks in England, a 64% higher number of confirmed NoV laboratory reports (hospital- and community-acquired) in England and Wales, and NoV-related deaths in elderly in Japan. The first molecular data uploaded to the international molecular surveillance database NoroNet from Australia, France, New Zealand and Japan indicate that this increase is associated with emergence of a new variant of genotype II.4 (GII.4). The first report of this variant was from Australia in March 2012 (personal communication P.A. White, September 2012), and the strain sequence was submitted to GenBank (accession number: JX459908.1). In the United States (US), the variant (named Sydney 2012) was detected in September 2012 in five of 22 (23%) laboratory-confirmed outbreaks, and in November in 37 of 71 (52%) laboratory-confirmed outbreaks (recorded in the US norovirus surveillance network CaliciNet)[4]. In two European countries that have not reported any indications of increased activity, the new variant has been found in outbreaks, two in Belgium (September and December 2012) and one in Denmark (November 2012). Other countries participating in NoroNet have not yet reported the new variant.

NoV is the predominant aetiological viral agent of acute gastroenteritis worldwide and is present throughout the year, but most prevalent in the winter season in temperate climates. In the last decade, strains belonging to NoV GII.4 have been responsible for the majority of outbreaks, as well as community cases of acute gastroenteritis. It has been suggested that hospitalisation and deaths occur more frequently during peak seasons associated with new NoV GII.4 variants[5-7]. Since 1995, new epidemic variants of GII.4 have emerged every two to three years, with population immunity and genetic drift as major evolutionary driving forces[8]. Emergence of new variants has been associated with increased NoV activity early in the season[9-11]. The newly found NoV GII.4 Sydney 2012 variant has evolved from previous NoV GII.4 variants (figure 1) and will be described in detail elsewhere. Briefly, the NoV GII.4 Sydney 2012 variant has a common ancestor with the dominant NoV GII.4 variants Apeldoorn_2007 and NewOrleans_2009, but is phylogenetically distinct. Amino acid changes

2.1
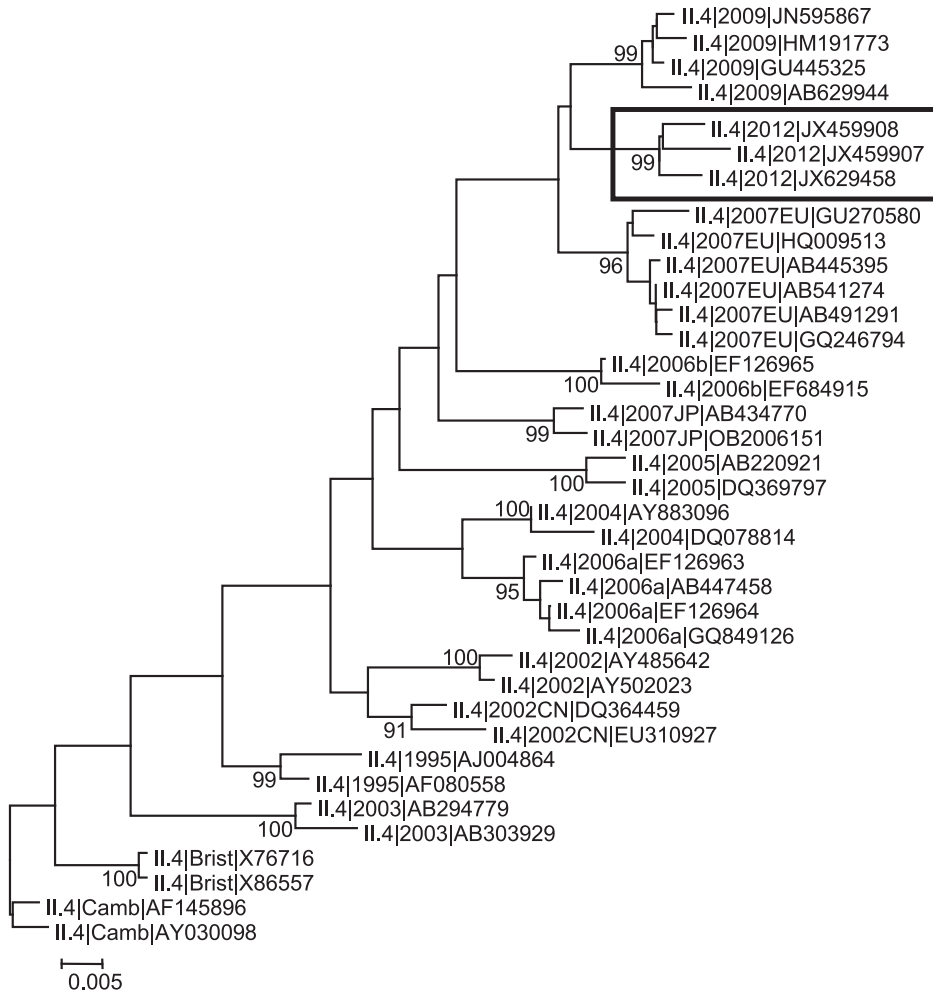
Figure 1 Neighbour-joining tree of norovirus GII.4 capsid amino acid sequences

are seen in the main epitopes located at the P2 domain, consistent with observations from prior epidemics. This may have led to an escape to existing herd immunity and might explain the observed increased outbreak activity.

The reference set of the Norovirus Typing Tool has been updated to correctly assign GII.4 Sydney 2012 sequences. This web-based tool (http://www.rivm.nl/mpf/norovirus/typingtool) is publicly available for genotyping of NoV sequences and was developed to facilitate standardisation of nomenclature[12].

Conclusion

Various countries around the globe have reported a higher incidence of NoV outbreaks or illness late 2012, and the first molecular data available via NoroNet suggests that this increase is related to emergence of a new variant of NoV GII.4. More data is needed to confirm the association between a higher NoV incidence and the new NoV GII.4 2012 variant. For this, we invite new members to join the NoroNet network (http://www.noronet.nl). Noronet is a worldwide network for NoV molecular and epidemiological surveillance, through which countries in Europe, Asia, and Australasia have shared NoV outbreak data, sequences, and other information. The NoroNet database, including analysis tools, is accessible for all NoroNet members.

With the early signs of a severe NoV season, healthcare institutions are advised to be prepared for NoV introductions. Outbreak management measures, like stringent hygiene measures and quarantine of infected cases, can help to reduce the size of outbreaks[13,14].

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2.1

Emergence of a novel GII.17 norovirus – End of the GII.4 era?

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Abstract

In the winter of 2014/15 a novel GII.P17-GII.17 norovirus strain (GII.17 Kawasaki 2014) emerged, as a major cause of gastroenteritis outbreaks in China and Japan. Since their emergence these novel GII.P17-GII.17 viruses have replaced the previously dominant GII.4 genotype Sydney 2012 variant in some areas in Asia but were only detected in a limited number of cases on other continents. This perspective provides an overview of the available information on GII.17 viruses in order to gain insight in the viral and host characteristics of this norovirus genotype. We further discuss the emergence of this novel GII.P17-GII.17 norovirus in context of current knowledge on the epidemiology of noroviruses. It remains to be seen if the currently dominant norovirus strain GII.4 Sydney 2012 will be replaced in other parts of the world. Nevertheless, the public health community and surveillance systems need to be prepared in case of a potential increase of norovirus activity in the next seasons caused by this novel GII.P17-GII.17 norovirus.

Introduction

In this issue of Eurosurveillance, observations from Japan are reported on an unusual prevalence of a previously rare norovirus genotype, GII.17, in diarrheal disease outbreaks at the end of the 2014/15 winter season[1], similar to what was observed for China[2, 3]. Norovirus is a leading cause of gastroenteritis[4]. Although the infection is self-limiting in healthy individuals, clinical symptoms are much more severe and can last longer in immunocompromised individuals, the elderly and young children[5, 6].

The Norovirus genus comprises seven genogroups (G), which can be subdivided in more than 30 genotypes[7]. Viruses belonging to the GI, GII and GIV genogroups can infect humans, but since the mid-1990s GII.4 viruses have caused the majority (ca 70–80%) of all norovirus-associated gastroenteritis outbreaks worldwide[8-10].

GII.4 viruses can continue to cause widespread disease in the human population because they evolve through accumulations of mutations into so-called drift variants that escape immunity from previous exposures[11]. Contemporary GII.4 noroviruses also demonstrate intra-genotype recombination near the junction of open reading frame (ORF) 1 and ORF2, which is likely to foster the emergence of novel GII.4 variants[12]. In addition, the binding properties of GII.4 viruses have altered over time, resulting in a larger susceptible host population[13].

Emergence and geographical spread of GII.17 genotype noroviruses

Viruses of the GII.17 genotype have been circulating in the human population for at least 37 years; the first GII.17 strain in the National Center for Biotechnology Information (NCBI) databank is from 1978[14]. Since then viruses with a GII.17 capsid genotype have sporadically been detected in Africa, Asia, Europe, North America and South America (Table, Figure 1). The virus appears to be clinically relevant, as it has been associated with acute gastroenteritis (AGE) in children and adults, and with chronic infection in an immunocompromised renal transplant patient[15] and a leukaemia patient (unpublished data). In the United States (US), only four GII.17 outbreaks were reported between 2009 to 2013 through CaliciNet, with a median of 11.5 people affected by each outbreak[16]. In Noronet, an informal international network of scientists working in public health institutes or universities sharing virological, epidemiological and molecular data on norovirus, GII.17 cases were also sporadically reported in Denmark and South Africa during this period[17].

Detection of the novel GII.17 virus in environmental samples

The novel GII.17 is the predominant genotype

▲ **Sporadic detection of GII.17 viruses from before the emergence of the novel GII.17 virus**

Sporadic detection of the novel GII.17 virus

Major outbreaks of the novel GII.17 virus
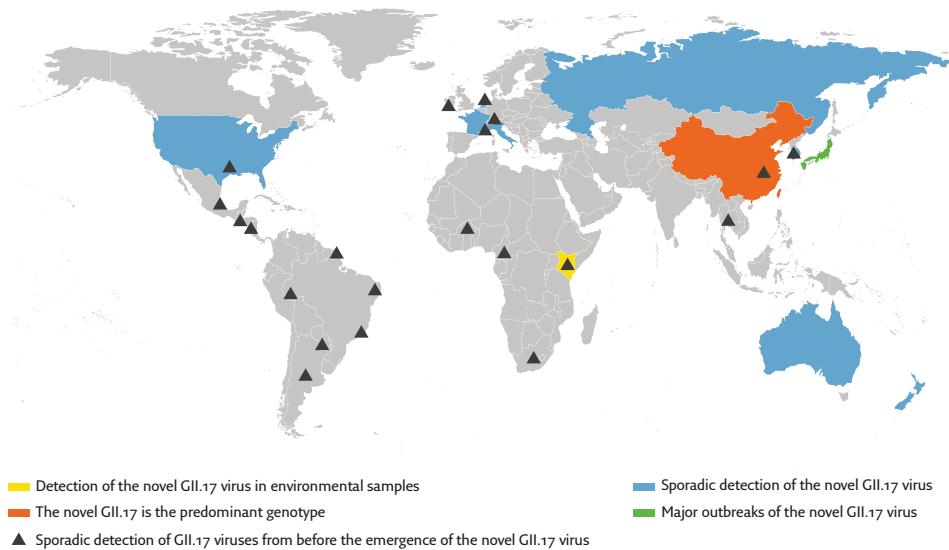
Figure 1 World map showing areas where GII.17 norovirus strains have been detected, 1978–2015

More widespread circulation of GII.17 was first reported for environmental samples in Korea from 2004 to 2006. This information was published in a report in 2010 by the Korean Food and Drug Administration (KFDA) and was cited by Lee *et al.*[18], but the original document describing this finding is not publicly available and there are no matching clinical reports. From 2012 to 2013 a novel GII.17 virus accounted for 76% of all detected norovirus strains in rivers in rural and urban areas in Kenya[19]. In the winter of 2014/15, genetically closely related GII.17 viruses were first detected in AGE outbreaks in the Guangdong province in China in schools, colleges, factories and kindergartens[2]. Sequence analyses demonstrated that 24 of the 29 reported outbreaks during that winter were caused by GII.17. A large increase in the incidence of AGE outbreaks was also reported; 29 outbreaks associated with 2,340 cases compared with nine outbreaks and 949 cases in the previous winter when GII.4 Sydney 2012 still was the dominant genotype[2].

During the same winter there was also an increase in outbreak activity in Jiangsu province, which could be attributed to the emergence of this novel GII.17[3]. This triggered us to investigate the prevalence of GII.17 in other parts of the world by means of a literature study and by inviting researchers collaborating within Noronet to share their data on GII.17. Currently, in Asia, in addition to Guangdong and Jiangsu[2, 3], the novel GII.17 is also the predominant genotype in Hong Kong (unpublished data) and Taiwan[20], while in Japan, a sharp increase

in the number of cases caused by this novel virus has been observed during the 2014/15 winter season[1]. Related viruses have been detected sporadically in the US[21] (http://www.cdc.gov/norovirus/reporting/calicinet/index.html), Australia, France, Italy, Netherlands, New-Zealand and Russia (unpublished data, www.noronet.nl) (Figure 1). In France the novel GII.17 virus appeared at the beginning of 2013, but since then, it has not resulted in an increase in AGE outbreaks as observed in China, nor replaced the predominant GII.4 in the last seasons (data not shown).

Based on sequence analyses of the ORF1-ORF2 junction region, most diagnostic real-time transcription polymerase chain reactions (PCRs) will be able to detect this novel GII.17 virus, but it is not known whether the same holds true for immunoassays. However, only a small portion of norovirus outbreaks are typed beyond the GI and GII classification, therefore it is possible that GII.17 is more prevalent than we currently suspect.

Phylogenetic analyses and molecular characterisation of the novel GII.17 viruses

Phylogenetic analysis of the viral protein 1 (VP1) of GII.17 strains in the NCBI database demonstrated at least two clusters, with the novel Asian GII.17 strains grouping together with the GII.17 strains detected in the surface water in Kenya (Figure 2,[21]) and in an outbreak in 2012 in Korea[22]. Although the novel GII.17 clusters away from previously identified GII.17 strains, the amino acids changes in VP1 are not sufficient to separate it into a different genotype. For only a limited number of GII.17 strains the full VP1 has been sequenced, which demonstrated three deletions and at least one insertion compared with previous GII.17 strains (comprehensive alignments are given in Fu *et al.* and Parra *et al.*[3, 21]). The majority of these changes could be mapped in or near major epitopes of the VP1 protein and potentially result in antigenic drift or altered receptor-binding properties[21]. Most publicly available GII.17 sequences only comprise the VP1, and most frequently the 5'-end of VP1 (C region), while most of the observed diversity within the GII.17 genotype is observed in the 3'-end of VP1 (D region)[23].

Previously, viruses with a GII.17 VP1 genotype contained a GII.P13 ORF1 genotype, although recombinants with an ORF1 GII.P16, GII.P3 and GII.P4 genotype have also been identified (Table). Sequence comparison showed that the ORF1 region of the novel GII.17 viruses was not detected before and cluster between GII.P3 and GII.P13 viruses[21]. Since this is the first orphan ORF1 sequence associated with GII.17, it has been designated GII.P17 according to the criteria of the proposal for a unified norovirus nomenclature and genotyping[24].

Table Overview of detected GII.17 norovirus strains worldwide, 1978–2015

Country	Geographical spread GII.17[c]	Year[a]	ORF1	ORF2	Study population	Percentage of typed strains/ outbreaks[b]	Suspected source of infection	Region sequenced	Accession number	references
French Guiana	Single location	1978	GII.P4	GII.17	Children with AGED	1 strain	-	Partial genome (7441 bp)	KC597139, JN699043	[14]
Brazil	Rio de Janeiro	1997 (1994-2008)	-	GII.17	Children with AGE	3/52 strains	-	5'-end ORF2 (300 bp)	JN600531	[31]
Kenya	Nairobi	1999-2000	-	GII.17	HIV positive children w/w.o. AGE	1/11 strains	-	5'-end ORF2 (309 bp)	KF279387	[32]
France	Briançon	2004	GII.P13	GII.17	Child with AGE	1 strain	-	Partial ORF1/2 (1361 bp)	EF529741	Data not shown
Paraguay	Asuncion	2004-2005	-	GII.17	AGE in children (<5 years)	5/29 strains	-	3'-end ORF2 (255 bp)	KC736582, KC736580, KC736578, KC736569	[33]
Brazil	States of Acre (Brazil)	2005 (2005-2009)	-	GII.17	AGE	2/62 strains	-	3'-end ORF2 (215 bp)	JN587118 JN587117	[34]
USA	Houston	2005	-	GII.17	AGE evacuees hurricane Katrina	predominant genotype in an outbreak	Sewage	ORF2 and 3 (2459 bp)	DQ438972	[35]
Argentina	Single location (Argentina)	2005-2006	-	GII.17	River samples	1/33 strains	-	-	-	[36]
Brazil	State of Rio de Janeiro	2005-2006 (2004-2011)	-	GII.17	Outbreaks of AGE	3/112 outbreaks	-	3'-end ORF2 (214 bp)	KJ179752, KJ179753, KJ179754	[37]
Nicaragua	Léon	2005-2006	-	GII.17	AGE	1 strain	-	5'-end ORF2 (244 bp)	EU780764	[26]
France	Sommières	2006	GII.P13	GII.17	AGE	1 strain	Foodborne	Partial ORF1/2 (1056 bp)	EF529742	Data not shown

2.2

Country	Location	Year		Genotype		Strains			Accession	Reference
Thailand	Lopburi	2006-2007	-	GII.17	AGE	2 strains	-	'5-end ORF2 (209 bp)	GQ325666, GQ325670,	[38]
China	Wuhan	2007 (2007-2010)	GII.P3	GII.17	AGE	1/488 strains	-	Partial ORF1/2 (1096 bp)	JQ751044	[39]
Mexico	Mexico City	2007	-	GiI.17	-		Waterborne	'5-end ORF2 (1337 bp)	JF976609	NCBI[d]
Switzerland	Zürich	2008	-	GII.17	Renal transplant patient	1/9 strains	-	ORF2 (1599 bp)	GQ266696	[15]
Nicaragua	Léon	2008	-	GII.17	AGE in children (<5 years)	2/38 strains	-	'5-end ORF2 (244 bp)	EU780764	[40]
Korea	Seoul	2010 (2008-2011)		GII.17	AGE	1/710 strains	-	'5-end ORF2 (209)	JQ944348	[41]
Brazil	Quilombola	2009 (2008-2010)	-	GII.17	Children (<10 years)	2/16 strains	-	'3-end ORF2 (215 bp)	JX047021, JX047022	[42]
Cameroon	Southwestern region of Cameroon	2009	GII.P3	GII.17	Healthy Children and HIV pos adults	4/15 strains	-	Partials ORF1/2 (1024 bp)	JF802504-JF802507	[43]
Guatemala	Tecpan	2009	-	GII.17	Children after waterborne outbreak	1/18 strains	Waterborne	-	-	[44]
Burkina Faso	Ouagadougou	2009-2010	-	GII.17	AGE in children (<5 years)	1/36 strains	-	5'-end ORF2 (287 bp)	JX416405	[27]
Netherlands	Single location Netherlands	2002-2007		GII.17	Nosocomial	3/264 strains	Nosocomial	-	-	[45]
South Korea	South Korea	2010	-	GII.17	Groundwater samples	2/7 strains	-	5'-end ORF2 (311 bp)	KC915021-KC915022	[18]
Ireland	Ireland	2010	-	GII.17	Influent waste water	4/24 strains	-	5'-end (302 bp)	JQ362530	[46]

2.2

Country	Location	Year		GII.17	Sample	Strains		Genome region	Accession	Ref
South Africa	South Africa	2010-2011	-	GII.17	Waste water	9/69 strains	-	5'-end ORF2 (305 bp)	KC495680, KC495686, KC495672-KC495674, KC495664, KC495657, KC495655, KC495640	[47]
Korea	Jinhae Bay	2010-2011	-	GII.17	Oysters	1 strain	-	-	-	[48]
Morocco	Oujda (Morocco)	2011	-	GII.17	AGE in children (<5 years)	1/42 strains	-	5'-end (205 bp)	KJ162374	[49]
South Africa	Johannesburg (South Africa)	2011	GII.P16	GII.17	AGE	-		Partial ORF1/2 (1010 bp)	KC962460	[50]
Cameroon	Limbe	2011-2012	GII.P3	GII.17	Healthy adults and Children	4/100 strains	-	Partial ORF1/2 (653 bp)	KJ946493	[51]
Kenya	Kenya	2012-2013	-	GII.17	Surface water	16/21 strains	-	5'-end ORF2 (306 bp)	KF916584-KF916585, KF808227-KF808254	[19]
Korea	Gyeonggi	2012	-	GII.17	AGE outbreak	1 strain	water-borne	5'-end (205 bp)	KC413386 KC413399-KC413403	[22]
China	Guangdong province	2014-2015	-	GII.17	AGE outbreaks	24/29 outbreaks	-	5'-end (249 bp)	KP718638-KP718738	[2]
USA	Gaithersburg	2014	GII.P17	GII.17	AGE in child of 3 years	1 strain	-	Partial genome 7527 bp)	KR083017	[21]
China	Jiangsu province	2014-2015	GII.P17	GII.17	Outbreaks of AGE	16/23 outbreaks	-	-	KR270442-KR270449	[3]
Japan	Japan	2014-2015	GII.P17	GII.17	Outbreaks of AGE	122/661 outbreaks	-	Partial genome (7534-7555 bp)	AB983218, LC037415, LC043139, LC043167, LC043168, LC043305	[1]

a   GII.17 detection year(s) with study years between brackets.

b   Either the percentage of strains that was typed as GII.17 or the percentage of outbreaks that was caused by GII.17 is given.

c   GII.17 detection location with study location between brackets (when different from GII.17 detection location)

d   acute gastroenteritis (AGE)
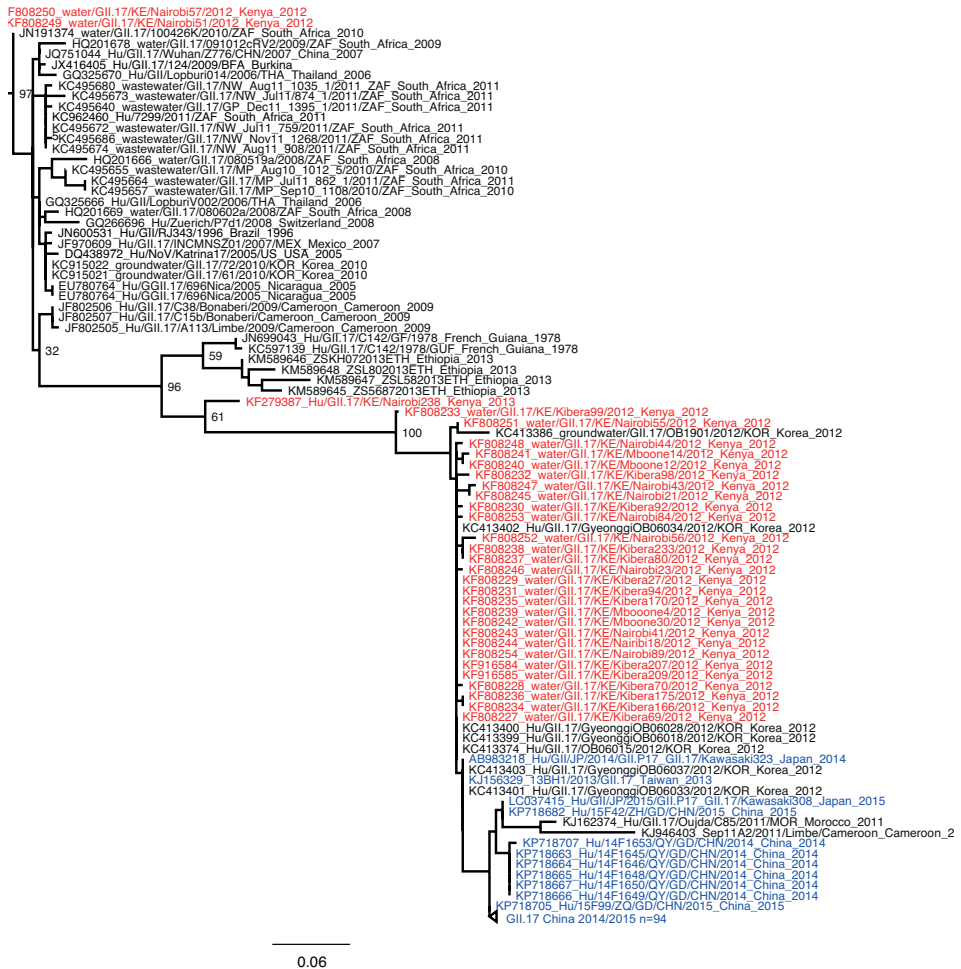
Figure 2 Unrooted maximum likelihood phylogenetic tree based on the 5'-end of virus protein 1 (VP1) sequences (C region) of GII.17 noroviruses, available from the National Center for Biotechnology Information (NCBI). The tree was estimated under the general time reversible model using PhyML. Bootstrap values above 70% are given. Sequences from Kenya are depicted in red and those from the recent outbreaks (2013–1015) reported in Asia in blue. The scale bar represents nucleotide substitutions per site.

The novel GII.17 virus was termed Kawasaki 2014 after the first near complete genome sequence (AB983218) submitted to GenBank. Noronet provides a publicly available and widely used tool for the typing of norovirus sequences (http://www.rivm.nl/mpf/norovirus/typingtool). This typing tool was updated to ensure correct classification of both ORF1 and ORF2 sequences of the newly emerged GII.P17-GII.17 viruses.

The acquisition of a novel ORF1 could potentially result in an increase in replication efficiency and may – in part – explain the increase of the AGE outbreak activity. Histo-blood group antigens (HBGAs) function as (co-) receptors for noroviruses. Alpha(1,2)fucosyltransferase 2 (FUT2) adds an alpha-1,2 linked fucose on HBGAs, and individuals lacking the FUT2 gene are referred to as 'non-secretors', while those with a functional FUT2 gene are called 'secretors'. Non-secretors have been shown to be less susceptible to infection with several norovirus genotypes[25]. In studies investigating the genetic susceptibility to norovirus genotypes, a secretor patient with blood type O Lewis phenotype Lea-b+ and a secretor patient with blood type B Lewis phenotype Lea-b- were positive for previously identified GII.17 viruses and no non-secretors were found positive[26, 27], suggesting that there could be genetic restrictions for GII.17 viruses in infection of humans. How the observed genetic changes have affected the antigenic and binding properties of the novel GII.17 strains, and hereby the susceptible host population, remains to be discovered.

Public health implications

Based on the emergence and spread of new GII.4 variants, we know that noroviruses are able to rapidly spread around the globe[28, 29]. The novel GII.17 virus has been detected in sporadic cases throughout the world, but until now it has not resulted in an increase in outbreak activity or replacement of GII.4 Sydney 2012 viruses outside of Asia. Following the patterns observed in the past years for GII.4 noroviruses and based on the data from China and Japan, an increase in norovirus outbreak activity can be expected if the currently dominant GII.4 is replaced by GII.17. Another possibility – however– would be some restriction to global expansion, as has been observed previously for the norovirus variant GII.4 Asia 2003[29]. Such restrictions could be due to differences in pre-existing immunity, but could also be the result of differences between populations in the expression of norovirus receptors[29]. Based on current literature on the novel GII.17 virus there is no indication that it will be more virulent compared with GII.4. Nevertheless, the public health community and surveillance systems need to be prepared in case of a potential increase of norovirus activity by this novel GII.17 virus.

Conclusions

Understanding the epidemiology of norovirus genotypes is important given the development of vaccines that are entering clinical trials. Current candidate vaccines have targeted the most common norovirus genotypes, and it remains to be seen if vaccine immunity is cross-reactive with GII.17 viruses[30]. Contemporary norovirus diagnostic assays may not have been developed to detect genotype GII.17 viruses since this genotype was previously only rarely found during routine surveillance. These assays need to be evaluated and updated if necessary to correctly diagnose norovirus outbreaks caused by the emerging GII.17 virus. Norovirus strain typing ideally should include ORF1 sequences and the variable VP1 'D' region as well as metadata on the host, like clinical symptoms, immune status and blood group. This will allow us to better study and monitor the genetic disposition, pathogenesis, evolution and epidemiology of this newly emerged virus.

2.2

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Analysis of norovirus molecular surveillance data collected through the NoroNet network, 2005 – 2016

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Abstract

Noroviruses are a common aetiology of acute gastroenteritis worldwide. Development of vaccines requires detailed understanding of global genetic diversity of noroviruses. This study describes trends in epidemiology and diversity based on global NoroNet surveillance data, and gives a future perspective on the global surveillance needs in light of these developments. The study analysed n=16635 norovirus sequences with associated epidemiological metadata, shared between 2005 and 2016 through NoroNet by partners from Europe, Asia, Oceania, and Africa. Sequences and epidemiological data were obtained from samples collected for outbreak investigations and diagnosis of sporadic gastroenteritis cases by clinical-, public health-, and food microbiology laboratories. During the study period, 26 different norovirus capsid genotypes circulated and 22 different recombinant genomes were found. The previously observed 2-3-year periodicity of emergence of genogroup II genotype 4 (GII.4) drift variants was not observed since 2012. Instead, the GII.4 Sydney capsid seems to persist through recombination, and we report a novel recombinant of GII.P16-GII.4 Sydney 2012 variant in Asia and Europe. The novel GII. P17-GII.17, first reported in Asia in 2014, has circulated widely in Europe. GII.4 viruses were more common in outbreaks in healthcare settings compared to other genotypes. Continuous changes in the global norovirus genetic diversity highlight the need for sustained global norovirus surveillance, including assessment of possible immune escape and evolution by recombination to provide a full overview of norovirus epidemiology for future vaccine policy decisions.

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Research in context

Evidence before this study

We searched Pubmed for articles published before 9th of July 2017 using keywords (worldwide OR global) AND norovirus AND genetic AND diversity in the title or abstract, and found 109 original research articles. The majority of studies reported on norovirus genetic diversity in a limited geographic area, timeframe, or focused on a single genotype. None of the studies presented long-term global norovirus diversity trends combined with epidemiological metadata, except one study focusing on the global norovirus diversity among oyster outbreaks.

Added value of this study

This study reports long-term global trends in norovirus genetic diversity combined with epidemiological metadata, obtained from reports from 19 countries across four continents/regions shared through a jointly owned database. It shows that multiple norovirus genotypes are co-circulating simultaneously with continuous and rapid changes in the norovirus genetic diversity worldwide, and with substantial regional differences, possibly reflecting differences in epidemiology, susceptibility, or both. We show differences in the preferred transmission route, preferred outbreak setting, and seasonal variation between norovirus genotypes. Finally, we discuss gaps in the norovirus surveillance and give recommendation for improvements to fulfil surveillance needs in light of vaccine development and other future interventions.

Implications of all the available evidence

Norovirus candidate vaccines are currently tested in clinical trials. This study shows that a future norovirus vaccine needs to induce broad protective immunity, or would need to be updated on a regular basis due to continuous and rapid changes in the norovirus genetic diversity. This study highlights the need for a global norovirus surveillance system using optimized sequencing protocols to monitor possible immune escape and evolution by recombination to provide data for vaccine updates. Future studies need to address the underlying factors for preferences in transmission routes, preferences in outbreak setting, and differences in seasonality among noroviruses.

Background

Acute gastroenteritis is the second greatest burden of all infectious diseases and norovirus is responsible for almost one fifth of all cases worldwide[1]. For healthy individuals, norovirus illness is typically self-limiting and of short duration, but risk groups like young children, elderly, and immunocompromised patients can suffer from prolonged symptoms[2]. In order to better understand the epidemiology and impact of norovirus and to identify (international) outbreaks, surveillance networks have been set up in some countries in the last two decades. These efforts have been challenging as norovirus surveillance is not mandatory in many countries, and if available does not always include genetic data. Despite these challenges, collaborative studies have identified international food-borne outbreaks, and substantially increased our knowledge on the norovirus diversity and antigenic evolution with the voluntary adoption of sequence-based typing[3, 4]. The genus *Norovirus* is highly diverse and divided in seven genogroups (G) of which GI, GII, and GIV have been found among humans. Genogroups are further subdivided in more than 40 genotypes[5]. The epidemiology and human health impact are strongly shaped by norovirus evolution through recombination or accumulation of mutations, known as genetic drift[6]. To capture this diversity, norovirus nomenclature is based on two parameters describing the genetic lineages of the gene encoding the viral polymerase (ORF1) and the capsid protein (ORF2). Polymerase genotypes are distinguished from capsid genotypes by a P in their name (e.g. GII.P4). This dual typing approach allows for tracking of noroviruses, including recombinant forms[7]. In 2002, an informal international data sharing network was established to study noroviruses and their diversity in relation to human health impact[8]. The work from NoroNet has contributed to the understanding that noroviruses from different genetic lineages may behave differently. Genogroup II genotype 4 (GII.4) has been the predominant strain globally and responsible for approximately 70% of outbreaks since the start of NoroNet[9-11]. The antigenicity of the capsid surface alters in a stepwise manner by selection of variants under the pressure of population immunity – a process called epochal evolution[3]. In addition, frequent exchanging of genes (recombination) results in emergence of novel noroviruses. There is currently no licensed norovirus vaccine on the market, but potential candidates have been tested in phase I and II clinical trials[12, 13]. Vaccine design is complicated by the large antigenic variation within the genus, and is currently targeting most commonly found genotypes. In view of the above, most likely, a future vaccine would need to be updated on a regular basis given the flexibility of norovirus to escape natural infection-derived population immunity, hence requiring improved coverage of surveillance[14]. We analysed whether and how data obtained via the NoroNet surveillance network

can be used to address the following outstanding questions regarding norovirus molecular epidemiology:

1. What are the trends in genomic diversity, recombination, and norovirus reporting?
2. Is there evidence for differences by genogroup / genotype in region, setting, and mode of transmission?
3. Where do new variants of norovirus emerge and can emerging variants be predicted from globally linked surveillance data?

Methods

NoroNet surveillance network

NoroNet links clinical-, public health-, and food microbiology laboratories willing to share norovirus molecular and epidemiological data on outbreaks and sporadic cases, and has been in existence since the mid-1990s[8, 10, 15]. The network started as EU funded network in 1999, continuing since 2002 as global NoroNet[8]. A jointly owned web-based database with online analysis tools was developed in which participants share and compare their data. Participation is on a give and take basis and partners have signed a code of conduct on uses of the data, after which they are granted full access to the data. Partners are expected to contribute to joint reports, and the joint database has been used for in depth studies following approval of partners.

Samples and study area

Specimens were obtained for the purpose of outbreak investigations and diagnosis of sporadic gastroenteritis cases. All RT-PCR positive cases confirmed by sequencing can be shared via NoroNet. Data from partners with less than 50 submitted sequences during the study period were excluded. Based on these criteria, the study included norovirus sequences obtained from samples collected in 19 countries: Austria, Belgium, China, Denmark, Finland, France, Germany, Hungary, Ireland, Italy, Japan, the Netherlands, New Zealand, Russia, Slovenia, South Africa, Spain, Sweden, and the United Kingdom. Less than 50 entries had been obtained from partners in Australia, Chile and Norway.

Data analysis

All entries submitted from January 1st 2005 to November 17th 2016 were downloaded on November 18th 2016. Records from non-human origin, without sample date or with a sample date prior to 2005 were removed from the analysis. Norovirus sequences were genotyped by the online norovirus typing tool[16]. Sequences overlapping the ORF1/ORF2 for which ORF1 and ORF2

genotypes could be assigned were analysed separately. All available sequences in the NoroNet database, including those before 2005, were used for the analysis of first reports of emerging GII.4 variants. The Maximum likelihood trees were inferred with PhyML version 3.1, using the general time reversible (GTR) nucleotide substitution model with a proportion of invariant sites and a Γ distribution of among-site rate variation[17].
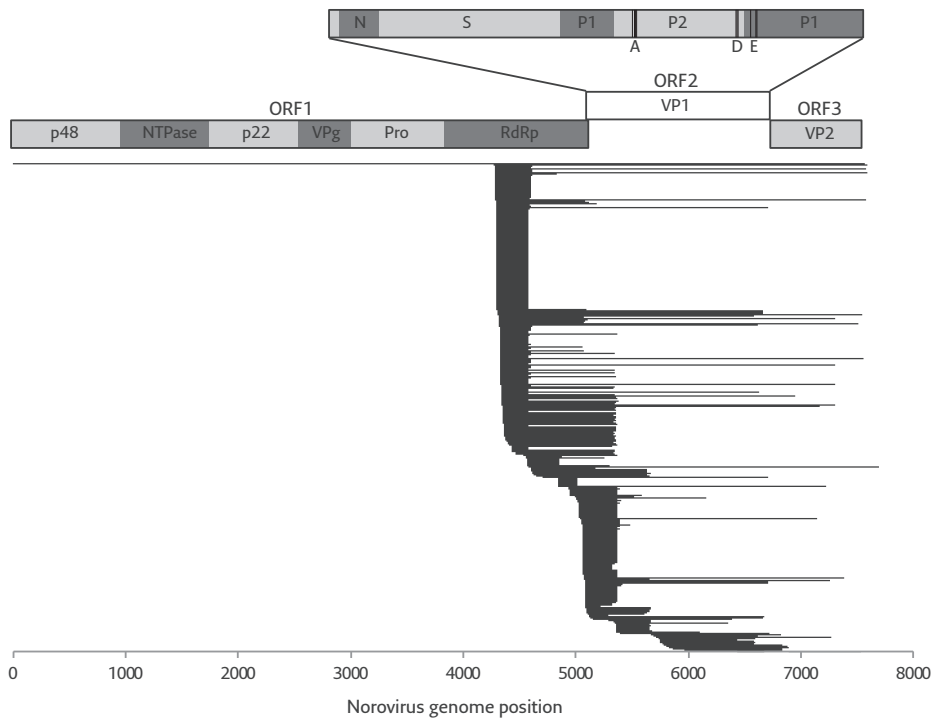
Figure 1 Position of 16628 sequence reads on the norovirus genome. Each sequence represents a line in the figure. Boxes above the graph represent the norovirus open reading frames (ORFs) of reference GII.Pe-GII.4 Sydney 2012 (Genbank accession: JX459908). ORF1 encodes for a polyprotein that is post-translationally cleaved by the virus-encoded protease (Pro) into six non-structural proteins (p48, NTPase, p22, VPg, Pro, and RNA-dependent RNA polymerase (RdRp)). ORF2 encodes for the major capsid protein (VP1) which consists of a shell (S) and protruding domains P1 and P2 with antigenic epitopes A, D, and E. ORF3 encodes for the minor capsid protein VP2.

Figure 2A (ORF1)

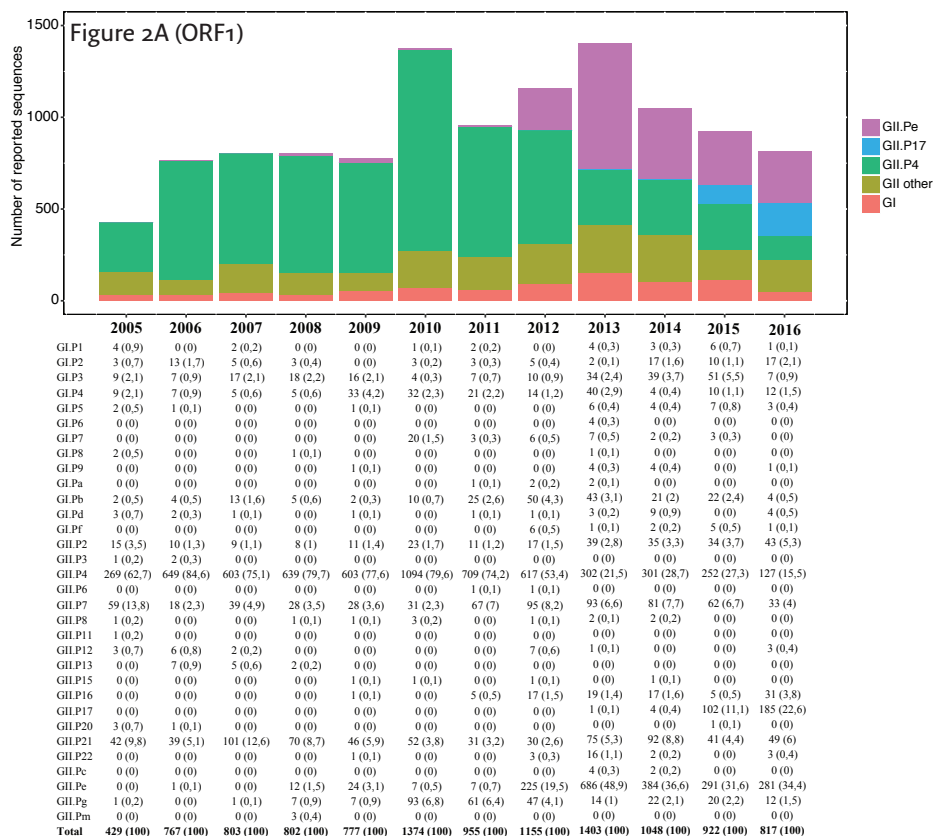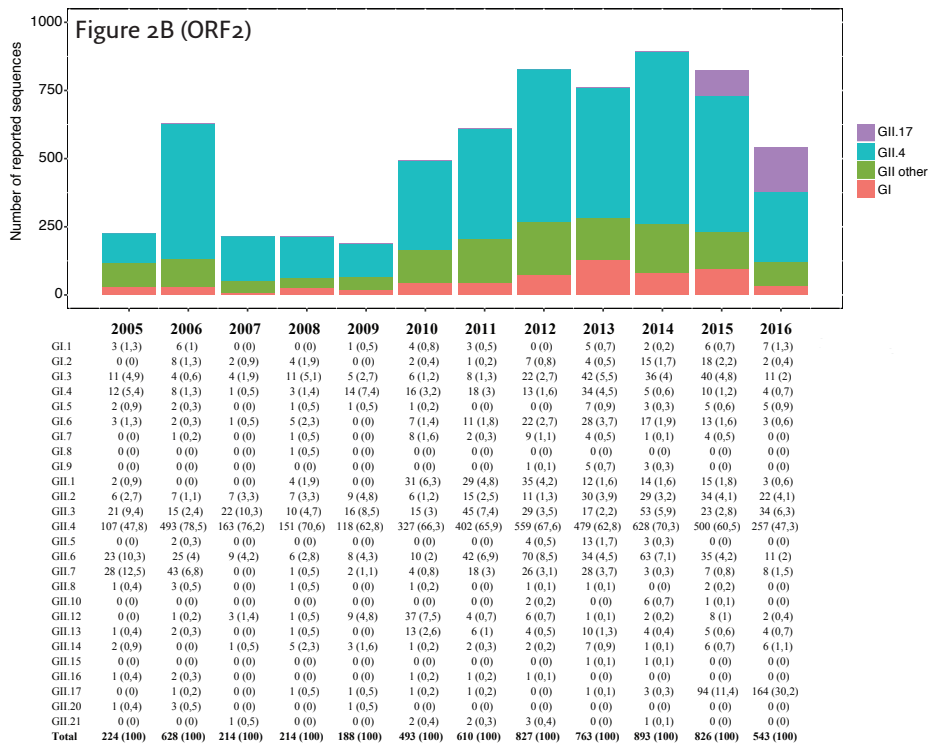|	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016
GI.P1	4 (0,9)	0 (0)	2 (0,2)	0 (0)	0 (0)	1 (0,1)	2 (0,2)	0 (0)	4 (0,3)	3 (0,3)	6 (0,7)	1 (0,1)
GI.P2	3 (0,7)	13 (1,7)	5 (0,6)	3 (0,4)	0 (0)	3 (0,2)	3 (0,3)	5 (0,4)	2 (0,1)	17 (1,6)	10 (1,1)	17 (2,1)
GI.P3	9 (2,1)	7 (0,9)	17 (2,1)	18 (2,2)	16 (2,1)	4 (0,3)	7 (0,7)	10 (0,9)	34 (2,4)	39 (3,7)	51 (5,5)	7 (0,9)
GI.P4	9 (2,1)	7 (0,9)	5 (0,6)	5 (0,6)	33 (4,2)	32 (2,3)	21 (2,2)	14 (1,2)	40 (2,9)	4 (0,4)	10 (1,1)	12 (1,5)
GI.P5	2 (0,5)	1 (0,1)	0 (0)	0 (0)	1 (0,1)	0 (0)	0 (0)	0 (0)	6 (0,4)	4 (0,4)	7 (0,8)	3 (0,4)
GI.P6	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	4 (0,3)	0 (0)	0 (0)	0 (0)
GI.P7	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	20 (1,5)	3 (0,3)	6 (0,5)	7 (0,5)	2 (0,2)	3 (0,3)	0 (0)
GI.P8	2 (0,5)	0 (0)	0 (0)	1 (0,1)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0,1)	0 (0)	0 (0)	0 (0)
GI.P9	0 (0)	0 (0)	0 (0)	0 (0)	1 (0,1)	0 (0)	0 (0)	0 (0)	4 (0,3)	4 (0,4)	0 (0)	1 (0,1)
GI.Pa	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0,1)	2 (0,2)	2 (0,1)	0 (0)	0 (0)	0 (0)
GI.Pb	2 (0,5)	4 (0,5)	13 (1,6)	5 (0,6)	2 (0,3)	10 (0,7)	25 (2,6)	50 (4,3)	43 (3,1)	21 (2)	22 (2,4)	4 (0,5)
GI.Pd	3 (0,7)	2 (0,3)	1 (0,1)	0 (0)	1 (0,1)	0 (0)	1 (0,1)	1 (0,1)	3 (0,2)	9 (0,9)	0 (0)	4 (0,5)
GI.Pf	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	6 (0,5)	1 (0,1)	2 (0,2)	5 (0,5)	1 (0,1)
GII.P2	15 (3,5)	10 (1,3)	9 (1,1)	8 (1)	11 (1,4)	23 (1,7)	11 (1,2)	17 (1,5)	39 (2,8)	35 (3,3)	34 (3,7)	43 (5,3)
GII.P3	1 (0,2)	2 (0,3)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
GII.P4	269 (62,7)	649 (84,6)	603 (75,1)	639 (79,7)	603 (77,6)	1094 (79,6)	709 (74,2)	617 (53,4)	302 (21,5)	301 (28,7)	252 (27,3)	127 (15,5)
GII.P6	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0,1)	1 (0,1)	0 (0)	0 (0)	0 (0)	0 (0)
GII.P7	59 (13,8)	18 (2,3)	39 (4,9)	28 (3,5)	28 (3,6)	31 (2,3)	67 (7)	95 (8,2)	93 (6,6)	81 (7,7)	62 (6,7)	33 (4)
GII.P8	1 (0,2)	0 (0)	0 (0)	1 (0,1)	1 (0,1)	3 (0,2)	0 (0)	1 (0,1)	2 (0,1)	2 (0,2)	0 (0)	0 (0)
GII.P11	1 (0,2)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
GII.P12	3 (0,7)	6 (0,8)	2 (0,2)	0 (0)	0 (0)	0 (0)	0 (0)	7 (0,6)	1 (0,1)	0 (0)	0 (0)	3 (0,4)
GII.P13	0 (0)	7 (0,9)	5 (0,6)	2 (0,2)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
GII.P15	0 (0)	0 (0)	0 (0)	0 (0)	1 (0,1)	1 (0,1)	0 (0)	1 (0,1)	0 (0)	1 (0,1)	0 (0)	0 (0)
GII.P16	0 (0)	0 (0)	0 (0)	0 (0)	1 (0,1)	0 (0)	5 (0,5)	17 (1,5)	19 (1,4)	17 (1,6)	5 (0,5)	31 (3,8)
GII.P17	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0,1)	4 (0,4)	102 (11,1)	185 (22,6)
GII.P20	3 (0,7)	1 (0,1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0,1)	0 (0)
GII.P21	42 (9,8)	39 (5,1)	101 (12,6)	70 (8,7)	46 (5,9)	52 (3,8)	31 (3,2)	30 (2,6)	75 (5,3)	92 (8,8)	41 (4,4)	49 (6)
GII.P22	0 (0)	0 (0)	0 (0)	0 (0)	1 (0,1)	0 (0)	0 (0)	3 (0,3)	16 (1,1)	2 (0,2)	0 (0)	3 (0,4)
GII.Pc	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	4 (0,3)	2 (0,2)	0 (0)	0 (0)
GII.Pe	0 (0)	1 (0,1)	0 (0)	12 (1,5)	24 (3,1)	7 (0,5)	7 (0,7)	225 (19,5)	686 (48,9)	384 (36,6)	291 (31,6)	281 (34,4)
GII.Pg	1 (0,2)	0 (0)	1 (0,1)	7 (0,9)	7 (0,9)	93 (6,8)	61 (6,4)	47 (4,1)	14 (1)	22 (2,1)	20 (2,2)	12 (1,5)
GII.Pm	0 (0)	0 (0)	0 (0)	3 (0,4)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Total	429 (100)	767 (100)	803 (100)	802 (100)	777 (100)	1374 (100)	955 (100)	1155 (100)	1403 (100)	1048 (100)	922 (100)	817 (100)

Figure 2 Number of reported ORF1 sequences (n=11252) stratified per genotype group, genotype, and year (**A**) and number of reported ORF2 sequences (n=6423) stratified per genotype group, genotype, and year (**B**). Note that n=1047 sequences overlapping ORF1/ORF2 are counted for both ORF1 and ORF2. Figure continues on the next page.

Results

Surveillance coverage

Sixteen countries (Austria, Belgium, Denmark, Finland, France, Germany, Hungary, Italy, the Netherlands, Spain, China, Japan, South Africa, Sweden, United Kingdom, Russia) submitted norovirus sequences in five or more successive years of which six countries submitted sequences during the entire study period (Finland, France, Germany, Hungary, Italy, and the Netherlands). The NoroNet surveillance network is well represented in Europe and has a smaller number of collaborators in Asia, Oceania, and Africa (Supplementary Table 1).

Figure 2B (ORF2)

	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016
GI.1	3 (1,3)	6 (1)	0 (0)	0 (0)	1 (0,5)	4 (0,8)	3 (0,5)	0 (0)	5 (0,7)	2 (0,2)	6 (0,7)	7 (1,3)
GI.2	0 (0)	8 (1,3)	2 (0,9)	4 (1,9)	0 (0)	2 (0,4)	1 (0,2)	7 (0,8)	4 (0,5)	15 (1,7)	18 (2,2)	2 (0,4)
GI.3	11 (4,9)	4 (0,6)	4 (1,9)	11 (5,1)	5 (2,7)	6 (1,2)	8 (1,3)	22 (2,7)	42 (5,5)	36 (4)	40 (4,8)	11 (2)
GI.4	12 (5,4)	8 (1,3)	1 (0,5)	3 (1,4)	14 (7,4)	16 (3,2)	18 (3)	13 (1,6)	34 (4,5)	5 (0,6)	10 (1,2)	4 (0,7)
GI.5	2 (0,9)	2 (0,3)	0 (0)	1 (0,5)	1 (0,5)	1 (0,2)	0 (0)	0 (0)	7 (0,9)	3 (0,3)	5 (0,6)	5 (0,9)
GI.6	3 (1,3)	2 (0,3)	1 (0,5)	5 (2,3)	0 (0)	7 (1,4)	11 (1,8)	22 (2,7)	28 (3,7)	17 (1,9)	13 (1,6)	3 (0,6)
GI.7	0 (0)	1 (0,2)	0 (0)	1 (0,5)	0 (0)	8 (1,6)	2 (0,3)	9 (1,1)	4 (0,5)	1 (0,1)	4 (0,5)	0 (0)
GI.8	0 (0)	0 (0)	0 (0)	1 (0,5)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
GI.9	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0,1)	5 (0,7)	3 (0,3)	0 (0)	0 (0)
GII.1	2 (0,9)	0 (0)	0 (0)	4 (1,9)	0 (0)	31 (6,3)	29 (4,8)	35 (4,2)	12 (1,6)	14 (1,6)	15 (1,8)	3 (0,6)
GII.2	6 (2,7)	7 (1,1)	7 (3,3)	7 (3,3)	9 (4,8)	6 (1,2)	15 (2,5)	11 (1,3)	30 (3,9)	29 (3,2)	34 (4,1)	22 (4,1)
GII.3	21 (9,4)	15 (2,4)	22 (10,3)	10 (4,7)	16 (8,5)	15 (3)	45 (7,4)	29 (3,5)	17 (2,2)	53 (5,9)	23 (2,8)	34 (6,3)
GII.4	107 (47,8)	493 (78,5)	163 (76,2)	151 (70,6)	118 (62,8)	327 (66,3)	402 (65,9)	559 (67,6)	479 (62,8)	628 (70,3)	500 (60,5)	257 (47,3)
GII.5	0 (0)	2 (0,3)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	4 (0,5)	13 (1,7)	3 (0,3)	0 (0)	0 (0)
GII.6	23 (10,3)	25 (4)	9 (4,2)	6 (2,8)	8 (4,3)	10 (2)	42 (6,9)	70 (8,5)	34 (4,5)	63 (7,1)	35 (4,2)	11 (2)
GII.7	28 (12,5)	43 (6,8)	0 (0)	1 (0,5)	2 (1,1)	4 (0,8)	18 (3)	26 (3,1)	28 (3,7)	3 (0,3)	7 (0,8)	8 (1,5)
GII.8	1 (0,4)	3 (0,5)	0 (0)	1 (0,5)	0 (0)	1 (0,2)	0 (0)	1 (0,1)	1 (0,1)	0 (0)	2 (0,2)	0 (0)
GII.10	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	2 (0,2)	0 (0)	6 (0,7)	1 (0,1)	0 (0)
GII.12	0 (0)	1 (0,2)	3 (1,4)	1 (0,5)	9 (4,8)	37 (7,5)	4 (0,7)	6 (0,7)	1 (0,1)	2 (0,2)	8 (1)	2 (0,4)
GII.13	1 (0,4)	2 (0,3)	0 (0)	1 (0,5)	0 (0)	13 (2,6)	6 (1)	4 (0,5)	10 (1,3)	4 (0,4)	5 (0,6)	4 (0,7)
GII.14	2 (0,9)	0 (0)	1 (0,5)	5 (2,3)	3 (1,6)	1 (0,2)	2 (0,3)	2 (0,2)	7 (0,9)	1 (0,1)	6 (0,7)	6 (1,1)
GII.15	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0,1)	1 (0,1)	0 (0)	0 (0)
GII.16	1 (0,4)	2 (0,3)	0 (0)	0 (0)	0 (0)	1 (0,2)	1 (0,2)	1 (0,1)	0 (0)	0 (0)	0 (0)	0 (0)
GII.17	0 (0)	1 (0,2)	0 (0)	1 (0,5)	1 (0,5)	1 (0,2)	1 (0,2)	0 (0)	1 (0,1)	3 (0,3)	94 (11,4)	164 (30,2)
GII.20	1 (0,4)	3 (0,5)	0 (0)	0 (0)	1 (0,5)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
GII.21	0 (0)	0 (0)	1 (0,5)	0 (0)	0 (0)	2 (0,4)	2 (0,3)	3 (0,4)	0 (0)	1 (0,1)	0 (0)	0 (0)
Total	**224 (100)**	**628 (100)**	**214 (100)**	**214 (100)**	**188 (100)**	**493 (100)**	**610 (100)**	**827 (100)**	**763 (100)**	**893 (100)**	**826 (100)**	**543 (100)**

Number of reported sequences, sequence length and genome position

A median of 870 (IQR 345) ORF1 sequences and a median of 577 (IQR 594) ORF2 sequences was reported per year. Sequence reads had an average length of 351 bases and the majority of sequences were located in the RNA-dependent RNA polymerase region of ORF1 or 5' side of ORF2 (Figure 1). Only 2·7% of sequences covered the main antigenic sites located at the P2 domain of VP1. During the study period, 154 full VP1 sequences were reported including three full genome sequences (KC175323, KC631827, and KP998539). An increased number of reported ORF1 sequences was observed in years of or post introduction of new GII.4 variants (Den Haag 2006b in 2006, New Orleans 2009 in 2009, and Sydney 2012 in 2012) which could be primarily attributed to GII.P4 and GII.Pe (Figure 2A). The apparent decline in number of reported sequences in 2016 is an artefact due to the selection of sequences until November 18th 2016 and a submission delay.

Figure 3 ORF1 GII.P4 variant trends per year (n=8083, top) and ORF2 GII.4 variant trends per year (n=4184, bottom). The relatively high proportion of viruses/sequences typed as "other" in the oldest category of submissions is an artefact due to the typing tool that was used. This tool performs a phylogeny based assignment of norovirus sequences to genera, genotypes, and variants. For correct assignment of variants, the reference sequences need to be periodically updated, when a new variants arise. By focusing on correct assignment of recent sequences, older strains may then be labelled as "unknown" with the current version of the typing tool.

Norovirus diversity at the genotype level

The number of reported sequences and GI versus GII ratio per country was analysed to get a better understanding of the genogroup coverage and diversity (Supplementary Table 1). Overall, 1372 of 16635 (8·2%) sequences belonged to norovirus GI, 15256 of 16635 (91·7%) sequences belonged to GII, and 7 of 16635 (0·0%) sequences belonged to GIV.1. Austria reported the lowest GI proportion (3·2%) and Sweden the highest (22·3%) among European countries, while countries in Asia and South Africa only reported GII strains. Trends per genotype per year for GI and GII are shown in Figures 2A and 2B. The most consistently and commonly detected genotype was GII.P4 with 6125 of 11252

(54·8%) ORF1 sequences and 4184 of 6423 (65·1%) ORF2 sequences listed as GII.4 by the phylogeny based typing tool. The remaining ~40% is a diverse mixture of 31 ORF1 and 25 ORF2 genotypes with some genotypes only detected incidentally, while other genotypes were detected more often in some years.

Emergence of novel GII.17 genotype
NoroNet detected a sharp increase in the number of GII.P17 and GII.17 strains in 2015 – 2016 compared to previous years (Figure 2A and 2B). GII.P17 and / or GII.17 were widely detected among European countries (Belgium, Finland, France, Germany, Hungary, Italy, the Netherlands, Russia, and Slovenia) in 2015 – 2016, but not in all (Ireland, Spain, and United Kingdom) (Supplementary Table 2A and Supplementary Table 2B). The GII.P17 and GII.17 proportion of total number of sequences per country showed large variation among European countries (range 4·2 - 53·9% and 5·3 - 44·5%, respectively). GII.P17 and GII.17 were co-circulating with GII.P4, GII.Pe, and GII.4 strains in Europe, and were only more prevalent than GII.P4, GII.Pe, or GII.4 in France (ORF1) and Russia (ORF1 and ORF2). China and Japan submitted in total n=10 ORF1 and n=73 ORF2 sequences to NoroNet in 2015 - 2016, and China reported n=1 GII.17 strain.

Trends in GII.4 variants
The NoroNet GII.4 variant distribution time trends are shown in Figure 3. In 2006, GII.4 Hunter 2004 was replaced by GII.4 Den Haag 2006b, succeeded by GII.4 New Orleans 2009 and GII.4 Sydney 2012 in the Northern hemisphere winter seasons of 2009/2010 and 2012/2013, respectively. The GII.4 Sydney ORF2 variant circulated as recombinant with GII.Pe or GII.P4 New Orleans 2009 since it emerged in 2012, and has not (yet) developed a new ORF1 variant. The GII.4 New Orleans 2009 ORF2 variant almost disappeared as of 2013, while the corresponding GII.P4 New Orleans ORF1 variant was still widely detected due to recombination with the GII.4 Sydney 2012 ORF2 variant. The GII.4 variant group 'other' represents variants that were only detected with limited geographic distribution and at low level incidence or sequences that could not be typed to the variant level by the norovirus genotyping tool i.e. due to a short sequence length. Variants that were detected infrequently during the study period are: Camberwell 1994, Farmington Hills 2002, Asia 2003, Kaiso 2003, Yerseke 2006a, Apeldoorn 2007, and Osaka 2007. A novel GII.P16-GII.4 Sydney 2012 recombinant was detected in 2014 (n=2) (Germany and the Netherlands), not detected in 2015, and detected in Japan, China, and the Netherlands (n=13) in 2016 (see paragraph recombination for more information on the novel GII.P16-GII.4 Sydney 2012 recombinant).
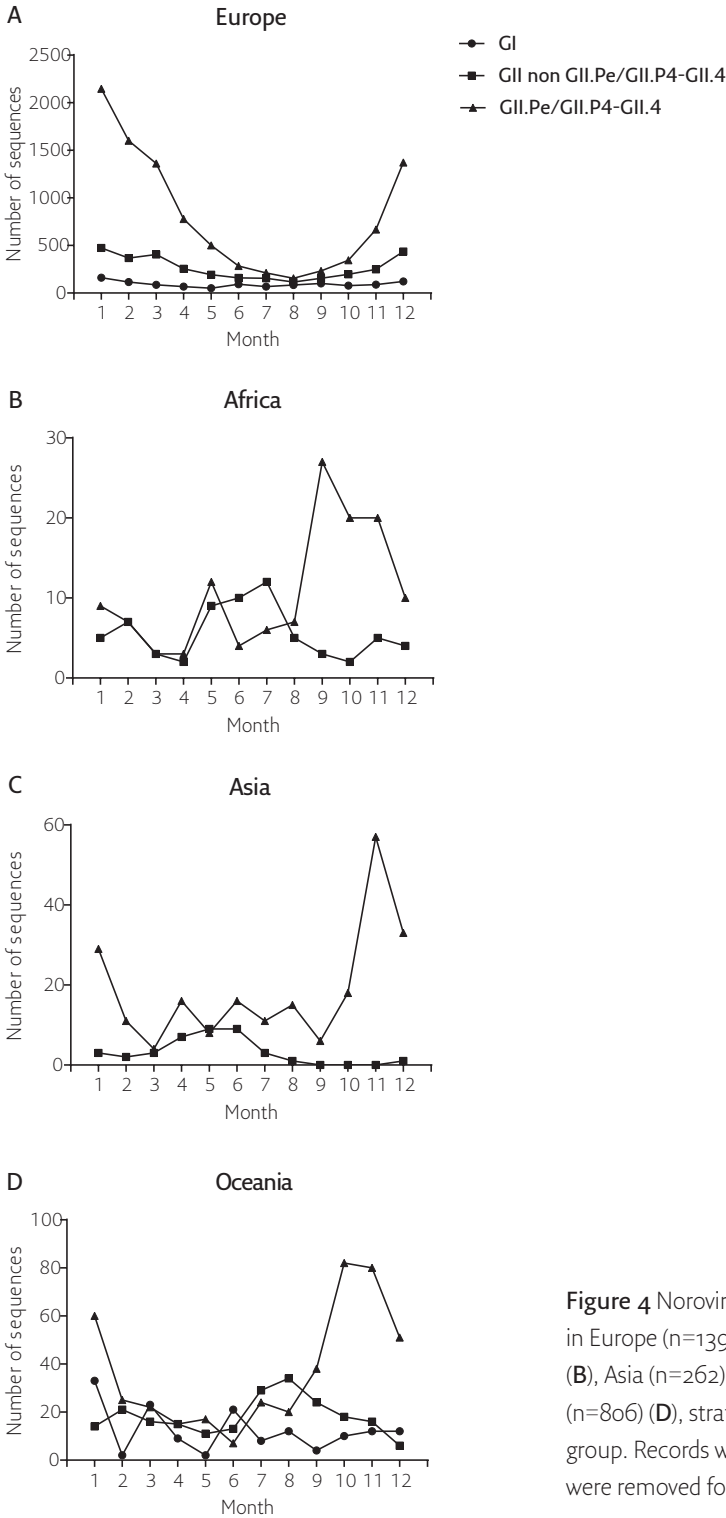
Figure 4 Norovirus seasonality patterns in Europe (n=13935) (**A**), Africa (n=195) (**B**), Asia (n=262) (**C**), and Oceania (n=806) (**D**), stratified per genotype group. Records without sample month were removed for this analysis.

A

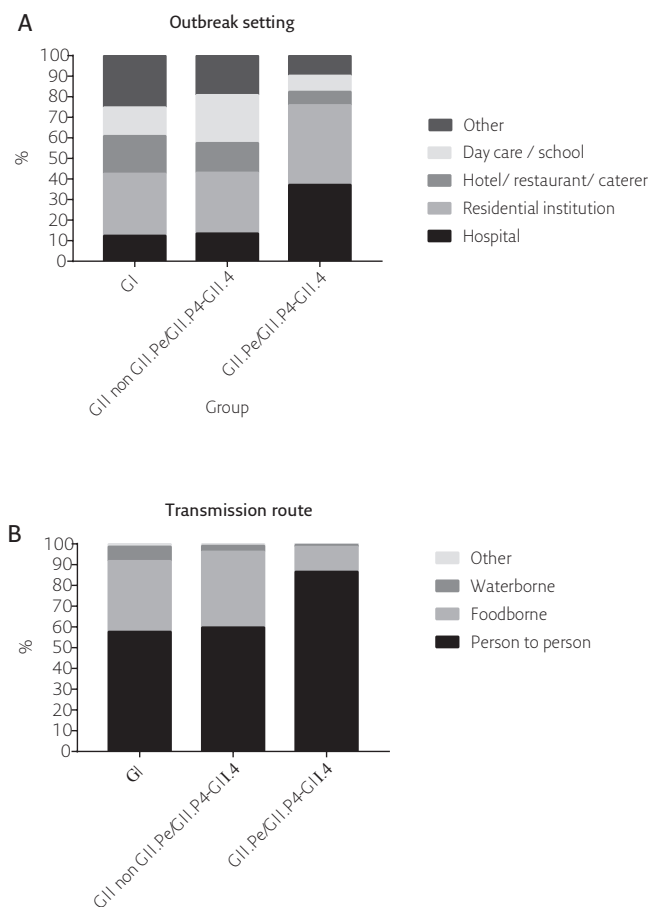### Outbreak setting

B

Transmission route

Figure 5 Norovirus transmission route (n=8772) (**A**) and suspected outbreak setting (n=6446) (**B**), stratified per genotype group. Records without known transmission route or suspected outbreak setting were removed. Outbreaks with suspected foodborne origin and subsequent person-to-person transmission were recoded as foodborne.

3

Origin of novel GII.4 drift variants

To assess when and where novel drift variants originate, we assessed the sampling date and country of origin of the first reported sequence of global drift variants (Supplementary Table 3). All assessed variants, except Hunter 2004, were detected 2-5 years before the global predominance of the particular strain, which may indicate that new drift variants were present at low levels in the population before their actual global emergence. Hunter 2004 was firstly detected in the Netherlands in the year of emergence 2004.

Recombination

To assess the influence of ORF1/ORF2 recombination on the norovirus diversity, we selected all sequences (n=1047) that were overlapping the ORF1/ORF2 junction and for which both ORF1 and ORF2 sides could be genotyped by the norovirus genotyping tool. 477 of 1047 (45·6%) sequences were assigned as a recombinant strain (Supplementary Table 4). No between genogroup recombination was observed. Remarkably, some polymerase types are more prone to recombine than others. Recombination within GII was most common: 457 recombinant sequences belong to GII of which GII.Pe–GII.4, GII.P21–GII.3, and GII.P7–GII.P6 are the most commonly detected recombinants. ORF2 GII.4 has been detected in combination with GII.P12, GII.P16, and GII.Pe. The GII.P12 recombinant was detected in 2005 – 2006 in combination with GII.4 Asia 2003. GII.P16 and GII.Pe are both only found in combination with GII.4 Sydney 2012 between 2014 and 2016 (data not shown). GII.P16 was found in combination with five different VP1 genotypes: GII.3, GII.4, GII.10, GII.12, and GII.13 which each form a separate clade in a maximum likelihood tree inferred from partial GII.P16 sequences (Supplementary Figure 1). Three variants of GII.4 Sydney are currently co-circulating, all resulting from recombination: GII.P4 Orleans 2009-GII.4 Sydney 2012, GII.Pe-GII.4 Sydney 2012 and GII.P16-GII.4 Sydney 2012. The antigenic regions in the capsid do not contain any amino acid changes compared to previously circulating GII.4 Sydney strains, although the VP1 sequences of GII.P16-GII.4 Sydney 2012 cluster separately from other GII. Pe-GII.4 Sydney strains (Supplementary Table 5 and Supplementary Figure 2).

Differences by season, region, setting, and mode of transmission

The European norovirus season coincides with the Northern Hemisphere winter season (Figure 4A). GII.Pe/GII.P4-GII.4 sequences show the clearest winter seasonality patterns while GI and GII non GII.Pe/GII.P4-GII.4 strains are more continuously present throughout the year, but never exceed the number of GII.Pe/GII.P4-GII.4 sequences. The rate of norovirus submissions in Africa (all reported by South Africa) shows an elevation in the months

September – November which coincides with the Southern Hemisphere spring season (Figure 4B). Asia (reported by China and Japan) shows an elevation of the norovirus incidence in the Northern Hemisphere winter season with the peak in November, two months earlier compared to Europe (Figure 4C). Oceania (reported by New Zealand) shows highest incidence in October and November (spring) (Figure 4D).

The suspected mode of transmission was reported for n=6446 entries: 77·4% person-to-person transmission (n=4990), 19·9% foodborne transmission (n=1280), 2·1% waterborne transmission, and 0·7% other transmission mode (n=133, n=43, respectively) (Figure 5A). GII.4 is relatively more often transmitted via person-to-person compared to other genotypes.

The setting of the norovirus outbreak was reported for n=8772 entries: 29·7% hospital setting (n=2603), 36·0% residential institution (n=3154), 9·3% hotel, restaurant or caterer (n=819), 11·8% day care or school (n=1039), 13·2% other (n=1157) (Figure 5B). The majority of sequences were derived from samples obtained in health care - or residential institutions. GII.4 was relatively more often detected in healthcare settings (hospitals and residential institutions) compared to non-GII.4 genotypes.

Discussion

Despite differences in norovirus surveillance among countries and a lack of it in many others, the current NoroNet system is able to observe global trends and major shifts in the genetic composition of the virus population at the level of genotype and variant, as was shown by this study and by others[6, 10, 18, 19].

The first question addressed in this study is about the trends in norovirus genomic diversity, recombination, and norovirus reporting. During the study period, we observed circulation of at least 26 ORF2 genotypes when looking at diversity of the capsid gene. The viral capsid contains epitopes that are targeted by protective antibody responses, and understanding this diversity is important for evaluation of candidate vaccines[20]. It was previously noted that increased notification reflect true increases in disease trends[18, 21]. Therefore, the observed increase in reported sequences post emergence of new GII.4 variants is probably related to an increase in norovirus activity. GII.4 Sydney 2012 is the predominantly detected variant worldwide since 2012 and, given the replacement cycle of two to three years shown for previous variants, a new antigenic variant has been anticipated for some years. This trend in antigenic evolution, however, was not observed in the period described here. Instead, viruses with GII.4

Sydney capsids, have evolved by recombination, suggesting that recombination somehow favours virus maintenance in the population. For GII.4, recombination has previously only been with the closely related sequence types GII. Pe and GII.P12, which are both suggested to be derived from an ancestor of GII.P4[22]. The drivers for emergence of recombinant genomes in a population previously exposed to the same capsid sequences remains to be understood. The novel recombinant GII.P16-GII.4 Sydney 2012 may have increased fitness due to changes in the RNA dependent RNA polymerase (RdRp) that alter the polymerase fidelity and interaction with VP1, leading to differences in replication and/or transmission efficiency[23-26].

In addition to the globally prevalent GII.4 viruses, recent studies from Asia reported a major shift in genotype composition from the predominant GII.4 to the novel GII.P17-GII.17 norovirus strain (GII.17 Kawasaki 2014) late 2014 and onwards[19, 27]. The number of detected GII.P17-GII.17 strains among Asian countries within our network was limited and likely caused by a filtered submission of the respective countries. The GII.P17-GII.17 strain was widely detected among most European countries in 2015 and 2016 and showed substantial differences in prevalence among countries. This strain has not (yet) fully replaced GII.4 strains.

The great genetic diversity of noroviruses is typically not considered in epidemiological or clinical studies, but may translate to differences in the epidemiology. Therefore, we compared distribution of reported modes of transmission and settings for the reported outbreaks by genotype (question 2). The most commonly reported transmission mode for the GII.4 outbreaks reported to NoroNet was person-to-person transmission and the most commonly reported setting was residential institution[10]. Underlying driving factors for these differences compared with other genotypes are unknown. We observed substantial regional variation in the norovirus genotype distribution possibly reflecting differences in epidemiology, susceptibility of the population, or both.

Norovirus surveillance is done on a voluntary basis since funding for the network is unavailable. This is reflected by unstable reporting behaviour of many countries and a potential bias in this study. A limitation of the NoroNet network is that unstandardized convenience sampling and irregular submission affects the ability of the network to robustly identify the effect of introduction of new genotypes and variants on the norovirus impact and severity. Another limitation of the study are the gaps on the surveillance map with missing or limited data from most countries in Africa, Middle East, North – and South America,

Oceania, and Asia. The USA and Australia do have norovirus surveillance, but use separate databases to store and analyse their data. Future integration of surveillance databases could help to improve our understanding of the norovirus (molecular) epidemiology.

A potential use of the NoroNet network is the identification of international outbreaks, which have been observed during periods of sustained funding[4, 28]. The currently provided sequence data can be used to genotype a virus to the level of genotype and variant, but is less suitable for phylogenetic analysis for the purpose of international outbreak investigations due to the lack of standardisation of sequencing protocols. The use of next generation sequencing is explored to allow whole genome sequencing as a new standard to overcome this problem[29-31]. Most countries currently upload data to the NoroNet database batch wise, which leads to a submission delay and identification of international outbreaks potentially months after their occurrence. Countries would need to upload data on a weekly basis to be able to set effective public health measures (i.e. withdraw of a contaminated food product from the market).

Norovirus vaccine candidates are currently in phase I and II trials and although vaccine cross-protection, efficacy, and effectiveness need to be evaluated, especially in vulnerable patient populations, it seems likely that a norovirus vaccine will be available in the near future. Such a vaccine will likely need to be updated on a regular basis due to escape of the virus from population immunity, especially by the predominant GII.4[32]. Essential data about the antigenic changes, especially those located in the P2 domain of the major capsid of the virus, can be obtained via a global surveillance system. As a minimum, a shared protocol for sequencing is needed, preferably including the ORF1 / ORF2 overlap to genotype both the viral RNA-dependent RNA polymerase and VP1, and to detect recombinant strains. A protocol for sequencing this particular region has been described[33]. In addition to this protocol, a subset of specimens could be monitored for changes in the antigenic regions using a protocol spanning the P domain of VP1. Whole genome sequencing via next generation sequencing techniques could replace both protocols and potentially provide a better insight in the evolution of the virus, including the not well studied VP2.

One of the major questions within the norovirus research field is whether we are capable of predicting emerging variants in the near future, the third and last question addressed in our study. All recent major drift variants were already circulating years before they became dominant as shown by this study and by others, suggesting early warning surveillance for variant emergence would be

possible[34]. If we assume that new variants develop in the human population and could emerge anywhere in the world, as shown by this study and by others, this would require a surveillance system with global coverage including large-scale genomics to capture both capsid diversity and recombination[35, 36]. A next step would be to predict antigenic properties from the genomic diversity, although this is likely to be challenging and requires development of phenotypic assays to assess antigenicity and immunity, similar to the model of the global influenza virus surveillance network. More research and new funding sources are needed to address these issues.

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Supplementary Table 1 Number of reported GI and GII sequences per continent/region and country

Continent	Country	GI (%)	GII (%)	Total
Europe	Austria	6 (3,2)	180 (96,8)	186
Europe	Belgium	41 (11,4)	319 (88,6)	360
Asia	China	0 (0)	142 (100)	142
Europe	Denmark	67 (10,4)	580 (89,6)	647
Europe	Finland	96 (8,5)	1037 (91,5)	1133
Europe	France	267 (8,2)	3004 (91,8)	3271
Europe	Germany	183 (16,4)	932 (83,6)	1115
Europe	Hungary	43 (5,2)	791 (94,8)	834
Europe	Ireland	11 (7)	147 (93)	158
Europe	Italy	23 (7,7)	276 (92,3)	299
Asia	Japan	0 (0)	293 (100)	293
Europe	Netherlands	327 (6)	5100 (94)	5427
Australia	New Zealand	148 (18,4)	658 (81,6)	806
Europe	Russia	23 (7,5)	283 (92,5)	306
Europe	Slovenia	15 (6,7)	209 (93,3)	224
Africa	South Africa	0 (0)	195 (100)	195
Europe	Spain	16 (5,5)	274 (94,5)	290
Europe	Sweden	69 (22,3)	241 (77,7)	310
Europe	United Kingdom	37 (5,9)	595 (94,1)	632

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Supplementary Table 2A Number of reported norovirus ORF1 sequences stratified per genogroup/genotype, country, and time

Country	2005–2014						2015–2016					
	GI (%)	GII other (%)	GII.P4 (%)	GII.P17 (%)	GII.Pe (%)	Total	GI (%)	GII other (%)	GII.P4 (%)	GII.P17 (%)	GII.Pe (%)	Total
Austria	6 (3,3)	28 (15,2)	145 (78,8)	0 (0,0)	5 (2,7)	184	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0
Belgium	14 (8,6)	45 (27,8)	62 (38,3)	0 (0,0)	41 (25,3)	162	3 (6,7)	12 (26,7)	14 (31,1)	5 (11,1)	11 (24,4)	45
China	0 (0,0)	0 (0,0)	0 (0,0)	1 (33,3)	2 (66,7)	3	0 (0,0)	8 (100)	0 (0,0)	0 (0,0)	0 (0,0)	8
Denmark	43 (8,3)	116 (22,4)	351 (67,6)	0 (0,0)	9 (1,7)	519	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0
Finland	83 (7,7)	108 (10,0)	862 (80,1)	0 (0,0)	23 (2,1)	1076	8 (30,8)	7 (26,9)	0 (0,0)	3 (11,5)	8 (30,8)	26
France	80 (7,0)	104 (9,1)	682 (59,4)	3 (0,3)	279 (24,3)	1148	17 (5,0)	34 (10,1)	71 (21,1)	143 (42,4)	72 (21,4)	337
Germany	81 (14,7)	167 (30,4)	249 (45,3)	0 (0,0)	53 (9,6)	550	20 (18,7)	31 (29,0)	11 (10,3)	11 (10,3)	34 (31,8)	107
Hungary	33 (4,5)	120 (16,3)	535 (72,9)	0 (0,0)	46 (6,3)	734	9 (11,8)	10 (13,2)	3 (3,9)	25 (32,9)	29 (38,2)	76
Italy	9 (7,0)	9 (7,0)	38 (29,7)	0 (0,0)	72 (56,3)	128	1 (4,2)	7 (29,2)	4 (16,7)	1 (4,2)	11 (45,8)	24
Japan	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	1 (100)	1	0 (0,0)	2 (100)	0 (0,0)	0 (0,0)	0 (0,0)	2
Netherlands	221 (5,3)	829 (19,7)	2516 (59,8)	1 (0,0)	642 (15,3)	4209	106 (10,3)	207 (20,2)	272 (26,5)	51 (5,0)	389 (38,0)	1025
New Zealand	71 (18,3)	102 (26,3)	47 (12,1)	0 (0,0)	168 (43,3)	388	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0
Russia	0 (0,0)	12 (92,3)	0 (0,0)	0 (0,0)	1 (7,7)	13	0 (0,0)	19 (21,3)	4 (4,5)	48 (53,9)	18 (20,2)	89
Slovenia	8 (13,3)	11 (18,3)	41 (68,3)	0 (0,0)	0 (0,0)	60	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0
South Africa	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0
Spain	6 (4,2)	22 (15,5)	114 (80,3)	0 (0,0)	0 (0,0)	142	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0
Sweden	4 (16,7)	12 (50)	4 (16,7)	0 (0,0)	4 (16,7)	24	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0
United Kingdom	21 (12,2)	11 (6,4)	140 (81,4)	0 (0,0)	0 (0,0)	172	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0

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Supplementary Table 2B Number of reported norovirus ORF2 sequences stratified per genogroup/genotype, country, and time

Country	2005-2014					2015-2016				
	GI (%)	GII other (%)	GII.4 (%)	GII.17 (%)	Total	GI (%)	GII other (%)	GII.17 (%)	GII.4 (%)	Total
Austria	0 (0,0)	1 (50,0)	1 (50,0)	0 (0,0)	2	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0
Belgium	14 (12,2)	24 (20,9)	77 (67,0)	0 (0,0)	115	10 (26,3)	10 (26,3)	2 (5,3)	16 (42,1)	38
China	0 (0,0)	36 (37,1)	60 (61,9)	1 (1,0)	97	0 (0,0)	0 (0,0)	1 (2,2)	44 (97,8)	45
Denmark	28 (18,8)	73 (49,0)	47 (31,5)	1 (0,7)	149	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0
Finland	5 (17,2)	3 (10,3)	21 (72,4)	0 (0,0)	29	0 (0,0)	2 (100)	0 (0,0)	0 (0,0)	2
France	130 (9,5)	230 (16,8)	1007 (73,5)	3 (0,2)	1370	40 (9,6)	67 (16,1)	151 (36,2)	159 (38,1)	417
Germany	63 (17,9)	118 (33,6)	170 (48,4)	0 (0,0)	351	19 (17,6)	30 (27,8)	11 (10,2)	48 (44,4)	108
Hungary	2 (3,8)	26 (50,0)	24 (46,2)	0 (0,0)	52	0 (0,0)	0 (0,0)	1 (4,5)	21 (95,5)	22
Ireland	4 (3,4)	14 (11,8)	101 (84,9)	0 (0,0)	119	7 (17,9)	8 (20,5)	0 (0)	24 (61,5)	39
Italy	15 (10,4)	23 (16,0)	106 (73,6)	0 (0,0)	144	0 (0,0)	7 (25,9)	3 (11,1)	17 (63,0)	27
Japan	0 (0,0)	0 (0,0)	265 (100)	0 (0,0)	265	0 (0,0)	0 (0,0)	0 (0,0)	28 (100)	28
Netherlands	26 (4,8)	84 (15,6)	428 (79,6)	0 (0,0)	538	35 (8,3)	73 (17,4)	37 (8,8)	275 (65,5)	420
New Zealand	77 (18,4)	115 (27,5)	226 (54,1)	0 (0,0)	418	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0
Russia	6 (6,4)	58 (61,7)	30 (31,9)	0 (0,0)	94	17 (15,5)	18 (16,4)	49 (44,5)	26 (23,6)	110
Slovenia	7 (5,7)	20 (16,3)	96 (78,0)	0 (0,0)	123	0 (0,0)	0 (0,0)	3 (7,3)	38 (92,7)	41
South Africa	0 (0,0)	65 (33,3)	128 (65,6)	2 (1,0)	195	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0
Spain	10 (7,2)	47 (34,1)	81 (58,7)	0 (0,0)	138	0 (0,0)	0 (0,0)	0 (0,0)	10 (100)	10
Sweden	65 (22,7)	116 (40,6)	103 (36,0)	2 (0,7)	286	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0
United Kingdom	37 (6,5)	76 (13,4)	456 (80,1)	0 (0,0)	569	0 (0,0)	11 (17,7)	0 (0,0)	51 (82,3)	62

Supplementary Table 3 First detections of global GII.4 drift variants

GII.4 variant	Year of emergence	First record ORF1	First ORF1 country	first record ORF2	First ORF2 country
Hunter 2004	2004	6-Apr-2004	The Netherlands	6-Apr-2004	The Netherlands
Den Haag 2006b	2006	14-Feb-2002	Germany	30-Sep-2003	Japan
New Orleans 2009	2009	12-Dec-2006	France	24-Apr-2009	South Africa
Sydney 2012	2012	-	-	Oct-2007	The Netherlands

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Supplementary Table 4 ORF1 / ORF2 combinations (n=1047) detected by NoroNet 2005 – 2016

	GI.1	GI.2	GI.3	GI.4	GI.5	GI.6	GII.1	GII.2	GII.3	GII.4	GII.5	GII.6	GII.7	GII.10	GII.12	GII.13	GII.14	GII.17	Total
GI.P1	9																		9
GI.P2		10																	10
GI.P3			26																26
GI.P4				15															15
GI.P5					9														9
GI.P7			1																1
GI.Pb						9													9
GI.Pd			10																10
GII.P2								12			1								13
GII.P4										441									441
GII.P7												27	9				6		42
GII.P12									3									1	4
GII.P16										16	2			5	3	5			31
GII.P17																		39	39
GII.P21									63						2				65
GII.P22										2				1					3
GII.Pc							3												3
GII.Pe								2		301									303
GII.Pg							8								6				14
Total	9	10	37	15	9	9	11	14	66	760	3	27	9	6	11	5	6	40	1047

Supplementary Table 5 Amino acid (aa) comparison of the blockade epitopes A, D, and E between reference GII.Pe-GII.4 Sydney 2012 and novel GII.P16-GII.4 Sydney 2012 recombinant strains.

Accession nr	Sample location	Sample date	GII.4 ORF2 variant	A 294	A 296	A 297	A 298	A 368	A 372	D 393	D 394	D 395	E 407	E 412	E 413
JX459908.1	Australia	Mar-12	GII.Pe – GII.4 Sydney 2012	T	S	R	N	E	D	G	T	T	S	N	T
JX459907.1	Australia	May-12	GII.Pe – GII.4 Sydney 2012	T	S	R	N	E	D	S	T	T	S	N	T
Outbreak number	Sample location	Sample date	Recombinant	294	296	297	298	368	372	393	394	395	407	412	413
OH16o02	Japan	Jan-16	GII.P16 – GII.4 Sydney 2012	T	S	R	N	E	D	S	T	T	S	N	T
CUHK-NS-886	Hong Kong	Jan-16	GII.P16 – GII.4 Sydney 2012	T	S	R	N	E	D	S	T	T	S	N	T
OC16023	Japan	Mar-16	GII.P16 – GII.4 Sydney 2012	T	S	R	N	E	D	S	T	T	S	N	T
CUHK-NS-937	Hong Kong	Mar-6	GII.P16 – GII.4 Sydney 2012	T	S	R	N	E	D	S	T	T	S	N	T
CUHK-NS-938	Hong Kong	Jul-16	GII.P16 – GII.4 Sydney 2012	T	S	R	N	E	D	S	T	T	S	N	T
5061600252	Netherlands	Jul-16	GII.P16 – GII.4 Sydney 2012	T	S	R	N	E	D	S	T	T	S	N	T
5061600253	Netherlands	Jul-16	GII.P16 – GII.4 Sydney 2012	T	S	R	N	E	D	S	T	T	S	N	T
5061600205	Netherlands	Apr-16	GII.P16 – GII.4 Sydney 2012	T	S	R	N	E	D	G	T	T	S	N	T
CUHK-NS-943	Hong Kong	Apr-16	GII.P16 – GII.4 Sydney 2012	T	S	R	N	E	D	S	T	T	S	N	T
CUHK-NS-1002	Hong Kong	Aug-16	GII.P16 – GII.4 Sydney 2012	T	S	R	N	E	D	S	T	T	S	N	T
CUHK-NS-1037	Hong Kong	Sep-16	GII.P16 – GII.4 Sydney 2012	T	S	R	N	E	D	S	T	T	S	N	T
CUHK-NS-1038	Hong Kong	Sep-16	GII.P16 – GII.4 Sydney 2012	T	S	R	N	E	D	S	T	T	S	N	T
CUHK-NS-1044	Hong Kong	Sep-16	GII.P16 – GII.4 Sydney 2012	T	S	R	N	E	D	S	T	T	S	N	T

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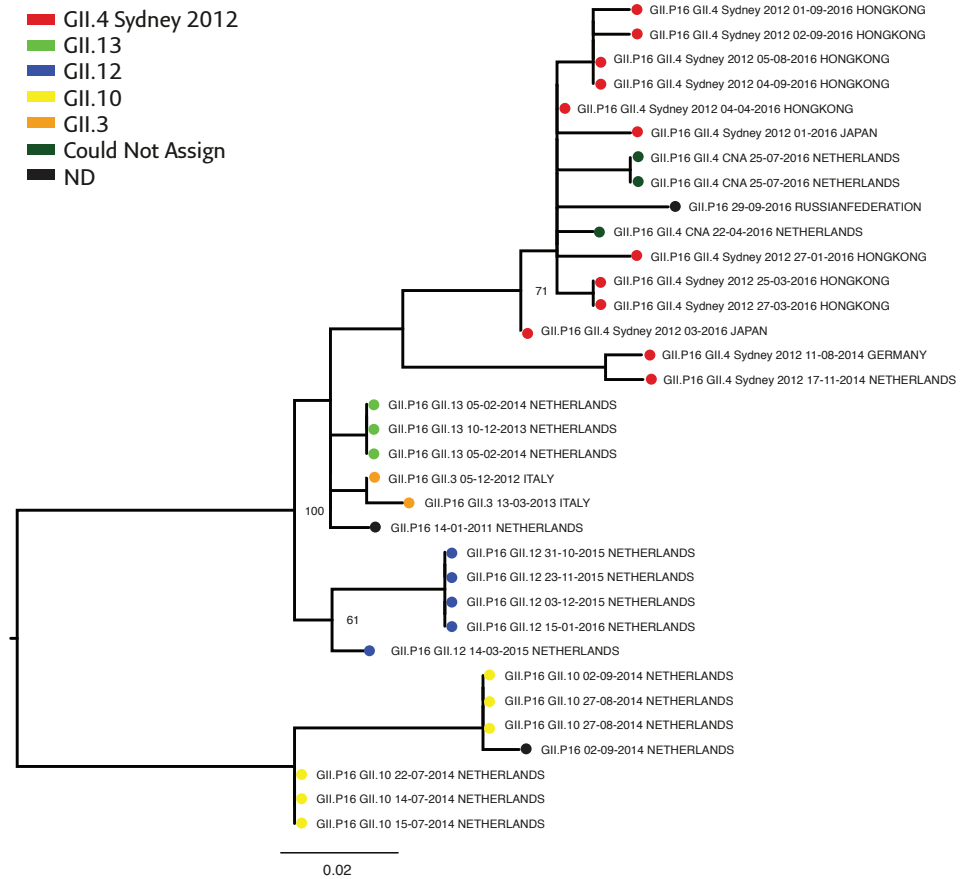
Supplementary Figure 1 Maximum likelihood tree for region B of ORF1 sequences displaying the genetic diversity of GII.P16 sequences that are found in combination with different VP1 sequences (used sequence length 289 nucleotides, n=34). GII.P16-GII.4 Sydney 2012 sequences are indicated in red.
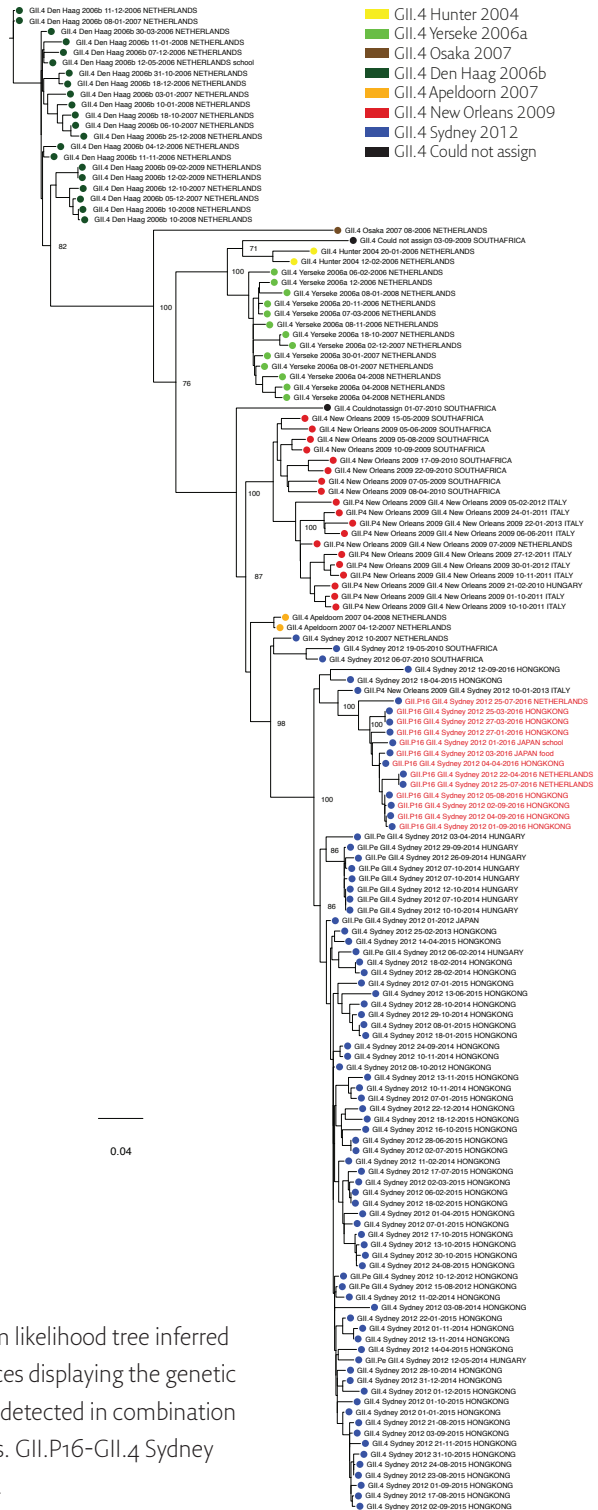
Supplementary Figure 2 Maximum likelihood tree inferred from all complete GII.4 VP1 sequences displaying the genetic diversity of GII.4 sequences that are detected in combination with different polymerase genotypes. GII.P16-GII.4 Sydney 2012 sequences are indicated in red.

Comparison of norovirus genogroup I, II, and IV seroprevalence among children in the Netherlands, 1963, 1983, and 2006

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Abstract

Noroviruses are a major cause of acute gastroenteritis worldwide and are a genetically diverse group of viruses. Since 2002, an increasing number of norovirus outbreaks have been reported globally, but it is not clear whether this increase has been caused by a higher awareness or reflects emergence of new genogroup II genotype 4 (GII.4) variants. In this study the hypothesis is tested that the norovirus prevalence has increased post 2002 and related to the emergence of GII.4. Sera from children aged <5 years of three Dutch cross-sectional population based cohorts collected in 1963, 1983, and 2006/2007 (n=143, n=130, and n=376, respectively) were tested for specific serum IgG by protein array using antigens to GII.4 and a range of other antigens representing norovirus GI, GII, and GIV genotypes. The protein array was validated by paired sera of norovirus infected patients and supernatants of B-cell cultures with single epitope specificity. Evidence for norovirus infection was found to be common among Dutch children in each cohort, but the prevalence towards different genotypes changed over time. At the genogroup level, GI seroprevalence decreased significantly between 1963 and 2006/2007, while a significant increase of GII and particularly genotype GII.4 specific antibodies was detected in the 2006/2007 cohort. There were no children with solely GII.4 antibodies in the 1963 cohort. This study shows that the high GII.4 norovirus incidence in very young children is a recent phenomenon. These findings are of importance for vaccine development and trials that are currently focussing mostly on GII.4 viruses.

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Introduction

Noroviruses belong to the family *Caliciviridae* and are a major cause of acute gastroenteritis in outbreaks and sporadic cases for all age groups worldwide[1]. Noroviruses are genetically highly diverse positive-stranded RNA viruses that can be divided into six genogroups (G) with a seventh genogroup recently proposed[2]. Viruses of GI, GII, and GIV are known to cause diarrheal disease in humans. The genogroups are further divided in approximately forty genotypes based on their phylogenetic clustering[3]. The lack of a robust cell culture system for norovirus has hampered the development of serological assays to study the population impact of individual norovirus genotypes. To be able to measure the immune response upon norovirus infection, ELISA assays based on virus-like particles (VLPs) produced through expression of the viral capsid protein (VP1) have been developed, but these assays cannot distinguish exposure to different genotypes due to high levels of cross-reactivity[4]. As a surrogate for virus neutralisation assays, assays have been developed to measure antibodies that block the binding of noroviruses to histo-blood group antigens[5]. These assays eliminate cross-reactivity observed for ELISA assays with VLPs, but are not suitable for population studies since they are difficult to standardise, time-consuming, and need large quantities of serum and VLP's. The VP1 consists of a conserved shell (S) domain and the more variable P domain that contains all immunogenic sites. Upon expression of the norovirus P domain P particles will be formed, which are very stable and immunologically relevant, but which contain less cross-reactive epitopes owing to the absence of the conserved S-domain, as evidenced from comparative immunization studies in mice[6, 7].

Since the start of norovirus surveillance in the mid-1990s, GII.4 have been the predominant genotype across the globe, responsible for 62% of outbreaks and the majority of endemic illness[1, 8, 9]. The norovirus GII.4 epidemiology has similarities to that of influenza A viruses, with new antigenic variants emerging every 2-3 years that replace the previously established variant, and herd immunity as the main evolutionary driving force[10, 11]. Since the mid-1990s, six GII.4 variants with pandemic spread have been recognized: US95/96, Farmington Hills 2002, Hunter 2004, Den Haag 2006b, New Orleans 2009, and Sydney 2012[12]. Little is known about norovirus genetic diversity before the mid-1990s since molecular techniques were not yet available and historical faecal collections are exceedingly rare. To our knowledge only one study has looked at the GII.4 molecular epidemiology before 1996 and has found GII.4 only in 9 of 48 (18.8%) faecal samples collected between 1974 and 1991[13]. Since the appearance of the GII.4 Farmington Hills 2002 variant, an increasing number of norovirus outbreaks have been reported compared to previous years,

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Table 1 Antibody titres (95% confidence interval) in pre and post infection sera.

Patient	Pre / post infection	Days relative to day of onset	Infecting genotype	GI.1	GI.2	GI.6	GI.8	GII.3	GII.4	GII.9
A	Pre	-32	Unknown history	20	20	20	20	20	20	20
A	Post	52	GII.4 Den Haag 2006b	20	20	20	20	411 (331-491)	5120	909 (710-1107)
B	Pre	-3	Unknown history	20	20	316 (281-352)	20	20	20	20
B	Post	46	GII.4 New Orleans 2009	20	20	145 (120-171)	20	86 (72-101)	5120	554 (495-613)
C	Pre	-1	Unknown history	20	20	20	20	136 (120-151)	20	20
C	Post	13	GII.3	20	20	20	20	2481 (2058-2904)	191 (165-217)	249 (223-275)
D	Pre	-4	Unknown history	125 (93-158)	87 (81-94)	100 (90-110)	20	20	250 (222-278)	63 (56-70)
D	Post	46	GI.6	20	20	553 (491-615)	20	20	127 (105-149)	20

but it is not clear whether this increase has been caused by a higher awareness or is an effect of the advancement of the emergence of antigenic drift variants of GII.4, or other evolutionary effects leading to increased fitness of these viruses at population level[14, 15]. Therefore, we wanted to test the hypothesis that the emergence of predomiant GII.4 viruses have been driving the increased norovirus burden since 2002. Sera from children under the age of 5 years were selected from three cross-sectional population based serum cohorts collected in 1963, 1983, and 2006/2007 and tested with a novel multiplex protein array to detect antibody responses to individual norovirus genotypes. Sera of young children were chosen to measure the impact of exposure to noroviruses in the first years of life. The protein array was validated using polyclonal rabbit sera, pre- and post- sera of norovirus infected individuals, and supernatants of B-cell cultures with single epitope specificity.

Materials and methods

Antigens

Norovirus P particles were used as antigens since they antigenically resemble native virions and can be produced in *E. coli* expression systems at relatively low costs as described[30, 31]. Furthermore P particles only contain the highly variable protruding (P) domain of the viral capsid protein (VP1), lack the more conserved S domain, and therefore contain less cross-reactive epitopes, as evidenced from comparative immunization studies in mice[6]. A GIV.1 VLP produced in insect cells was added to include antigens from all human genogroups. Since GIV is genetically not closely related to other human genogroups we did not expect cross-reactivity between these VLP and the P particles representing genotypes of GI and GII. We selected antigens representing common and rare genotypes as detected by the Noronet sequence database (Supplemental Table S1) (http://www.noronet.nl).

Norovirus protein microarrays for multiplex serology

Purified P particles and VLPs were diluted in protein array buffer (Maine Manufacturing, Sanford, Maine, USA) and protease inhibitor (BioVision, Mountain View, CA, USA), with final concentration of 1 mg/mL (determined by checkerboard titration, data not shown). Proteins were spotted in triplicate with two 333 pL spots onto 64-pad nitrocellulose coated slides (Oncyte avid, Grace bio-labs, Bend, OR, USA) using a non-contact Piezorray spotter (PerkinElmer, Waltham, MA, USA) as described[32]. Slides were incubated with Blotto blocking buffer (Thermo Fisher Scientific Inc., Rockford, MA, USA) to avoid non-specific nitrocellulose binding, and subsequently with serial 4-fold diluted human sera starting at a 1:40 dilution. Rabbit sera and B-cell supernatants were tested at a single dilution (1:20 for rabbit sera, 1:8 for B-cell supernatant pools, and 1:4 for individual cultures). After washing, slides were incubated with goat anti-human IgG or anti-rabbit IgG (Fc-fragment specific), conjugated with Alexa Fluor 647 fluorescent dye (Jackson Immuno Research, West Grove, PA, USA). Bound dye was quantified using a ScanArray Gx Plus microarray scanner (PerkinElmer).

Assay validation samples

Two rabbit polyclonal sera from animals immunized with recombinant norovirus GII.4 Den Haag 2006b or GIV VP1 protein (Immune Technology, New York, USA) were tested on the protein array to confirm that antigens remain intact on the platform and to test homologous versus heterologous antigen reactivity. Pre and post infection sera of RT-PCR confirmed norovirus patients were used for assay validation. These patients were infected with GII.4 Den Haag 2006b, GII.4 New Orleans 2009, GII.3, or GI.6 respectively (ages 5, 47, 17, and 12 y).

Use of the sera for assay validation was approved by the Erasmus MC medical ethical committee (MEC2013-082). Further validation of the array platform was performed by using sera (n = 40, storage at -20° C) and peripheral blood mononuclear cells (PBMCs) (storage at -135° C) obtained from two donors (10 years) who were sampled for a study on the memory immunity to *Bordetella pertussis* (ISRCTN64117538).

Quality control

The intra and inter assay variation was monitored by testing a serial diluted positive control serum consisting of pooled human sera reacting with high norovirus titre to antigens belonging to genogroup I and II. Samples tested on slides with a positive control deviated more than one 2-fold dilution step from the geometric mean titre were rejected from analysis. For the intra assay variation the positive control serum was tested 16 times on a single slide and to determine the inter assay variation the control serum was tested 44 times on multiple slides within 13 weeks. The quality of the GIV VLP was tested with a rabbit control serum (data not shown).

B-cell supernatants with single epitope specificity

B-cell supernatants with single epitope specificity were used to test the specificity of the protein array platform. B-cells were isolated, stimulated, and cultured in a limiting dilution assay with a slightly adjusted protocol as described before[16]. Briefly, PBMC were isolated within 24 h after venepuncture and stored at -135° C upon further use. The EasySep™ Human CD19 positive selection kit and EasySep magnet (Stemcell technologies, Cologne, Germany) were used to isolate the B-cells from the PBMC population. Purified B-cells were counted and re-suspended in 96-wells round bottom tissue culture plates with each well containing 500, 1000, or 4000 B-cells. Gamma irradiated CD40L-expressing murine fibroblast L cells were added in a concentration of 500 cells / well and 3 µg/ml CpG ODN2006 (Isogen Life Sciences, Utrecht, Netherlands), 10 ng/ml interleukin 2 (IL-2) (Miltenyi Biotec, Leiden, Netherlands) and 10 ng/ml IL-10 (BD Pharmingen, San Diego, USA) was added to promote cell division and antibody secreting cell (ASC) outgrowth. After 5 days of incubation at 37° C culture medium was refreshed and cytokines were replaced by 10 ng/ml IL-2 and 10 ng/ml IL-21(Invitrogen, Waltham, USA) to promote antibody production. Supernatants were harvested after 11-12 days, stored at -20° C, and *in vitro* IgG production was tested by total IgG ELISA (data not shown). Supernatants were subsequently tested against multiple antigens representing GI and GII genotypes on the protein array platform.

Study samples
A total of 649 serum samples from children <5 years of age collected in 1963, 1983, and 2006/2007 were included in the study, respectively n = 143, n = 130, and n = 376 (Table 3). Sera collected in 1963 and 1983 were obtained from a Dutch historical anonymous collection of serum samples, that had been collected for diagnostic purposes (not specifically for acute gastroenteritis), and stored at -20° C since then at Erasmus MC, Rotterdam[33]. Sera collected between February 2006 and June 2007 were obtained from a Dutch population based cross-sectional serosurvey[34]. The study was approved by the Medical Ethics Committee, Almere (ISRCTN 20164309).

Data- and statistical analysis
Serum titres were computed by fitting a 4-parameter log-logistic curve to 12 luminescence readouts (4 dilutions, each antigen tested in triplicate), using the point of inflection as titre as described[32]. A fixed minimum fluorescent signal of 3000 was chosen to reduce background reactivity and a fluorescent signal of 65535 was used as fixed maximum readout. Titres below the minimum dilution were set to a value half of the reciprocal minimal dilution and titres above the highest dilution were set to a value of 2 times the highest reciprocal serum dilution. Data of B-cell supernatants is shown as fluorescent signal (RFU) with the background signal subtracted since the supernatants were tested at a single dilution. Data analyses and statistical analysis were performed using R version 3.0.3. The chi-squared test for trend was performed to examine differences in seroprevalence rates between age groups and cohorts. A p-value ≤ 0.05 was considered to be significant. The Spearman correlation coefficient was used to assess potential cross-reactivity patterns between antibody titres detected against multiple norovirus genotypes.

Results
Array specificity
A novel multiplex norovirus P particle protein array was developed to be able to measure norovirus genotype specific antibodies. First, the specificity of the newly developed array was confirmed using polyclonal sera of two rabbits immunized with VP1 protein from GII.4 and GIV, respectively. Both the GII.4 and GIV rabbit sera reacted with high signal with the homologous antigen without significant cross-reaction with the heterologous antigens (data not shown). Next, we tested pre and post infection sera of four norovirus RT-PCR confirmed patients infected with GII.4 Den Haag 2006b, GII.4 New Orleans 2009, GII.3, and GI.6 respectively (Table 1, Supplemental Figure S1). All patients showed a more than fourfold increase to the homologous antigen showing that P particles
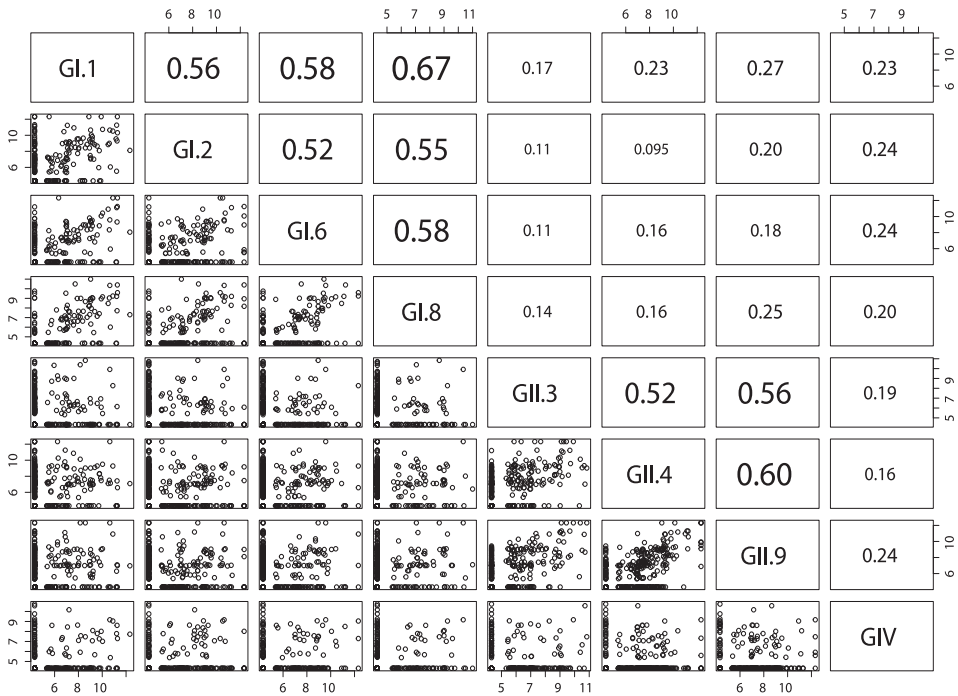
Figure 1 Multiple scatterplot log transformed titres of children <5 y (n=649) to determine potential cross-reactive patterns among P particle antigens on the protein array platform. In the top right triangle Spearman correlation coefficients are plotted between each antigen pair, with significant numbers being highlighted by enlargement.

in the array platform remain intact and are recognized by norovirus antibodies from patients. The pre infection sera of three patients (Table 1 patient B, C, and D) already showed reactivity, which was most likely caused by previous infections as expected since noroviruses are one of the most common infections during childhood. Antibody responses in the three GII infected patients (Table 1 patient A, B, and C) were exclusively observed for GII antigens, particularly the homologous antigen, with low levels of cross-reactivity or boosting of previous infections with heterologous GII antigens. The serum sample of the GI.6 infected patient (Table 1 patient D) showed a specific GI.6 reaction and did not bind to the heterologous GI and GII antigens.

Quality control
Using a positive control serum, consisting of pooled human sera, we determined that the average intra assay coefficient of variation (CV) for antibodies to individual P particle antigens was 9.0% (range 6.2-13.7%) and average CV inter

Table 2 B-cell supernatant profiles of two donors with a broadly reacting serum profile

		Donor	Cells	GI.1	GI.2	GI.6	GI.8	GII.3	GII.4	GII.9
Pool 1-12	1	8000	-	-	-	-	-	-	-	
Pool 13	1	8000	-	-	-	-	+	-	-	
Pool 14	1	8000	-	-	-	-	+++	-	-	
Pool 1-7	2	4000	-	-	-	-	-	-	-	
Pool 8	2	4000	-	-	-	-	-	++	-	
Pool 9	2	4000	-	+	-	+	-	-	-	
Pool 10	2	4000	-	+	-	-	-	-	-	
Pool 11	2	4000	-	-	+	-	-	-	-	
Pool 12	2	4000	+++	+++	+++	++	-	-	-	
	Pool 12 Culture 1-7	2	500	-	-	-	-	-	-	-
	Pool 12 Culture 8	2	500	+++	+++	+++	++	-	-	-
Pool 13	2	4000	-	+++	++	-	-	-	-	
	Pool 13 Culture 1-7	2	500	-	-	-	-	-	-	-
	Pool 13 Culture 8	2	500	-	+++	+	-	-	-	-
Pool 14	2	4000	-	-	-	-	-	+++	-	
Pool 15	2	4000	-	+	-	-	++	-	-	
Pool 16	2	4000	++	++	++	++	-	-	-	

Symbols -, +, ++, +++ represent respectively a fluorescent signal of 0 – 500, 501 – 5000, 5001 – 30000, >30000

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assay variation 25.7% (range 16.9-36.0%). The VLP GIV was not included in this analysis since the human control serum was not reactive to this antigen. The potential degradation of antibodies over time was monitored by comparing the magnitude of titres among cohorts. High antibody titres against individual antigens were observed in all three cohorts indicating that the serum antibodies were not degraded (Supplemental Figure S2).

Potential cross-reactivity of antibodies towards the antigens on the array
Sera of children below 5 years of age (n=649) were assessed for potential cross-reactivity patterns by the Spearman correlation coefficient and plotting of log transformed titres between each possible antigen pair (Figure 1). Antibodies directed to antigens belonging to different genogroups typically did not show significant cross-reactivity in this age group, with a low correlation coefficient

Table 3 Age stratified number of sera tested and percentage of sera with reactivity to any norovirus antigen.

Age group	1963		1983		2006/2007	
	n sera tested	% sera norovirus positive	n sera tested	% sera norovirus positive	n sera tested	% sera norovirus positive
<1	25	72.0	20	70.0	110	42.7
1-<2	32	50.0	23	56.5	57	64.9
2-<3	25	72.0	34	67.6	74	63.5
3-<4	27	66.7	24	79.2	75	85.3
4-<5	34	85.3	29	75.9	60	85.0
Total	143	69.2	130	70.0	376	65.4
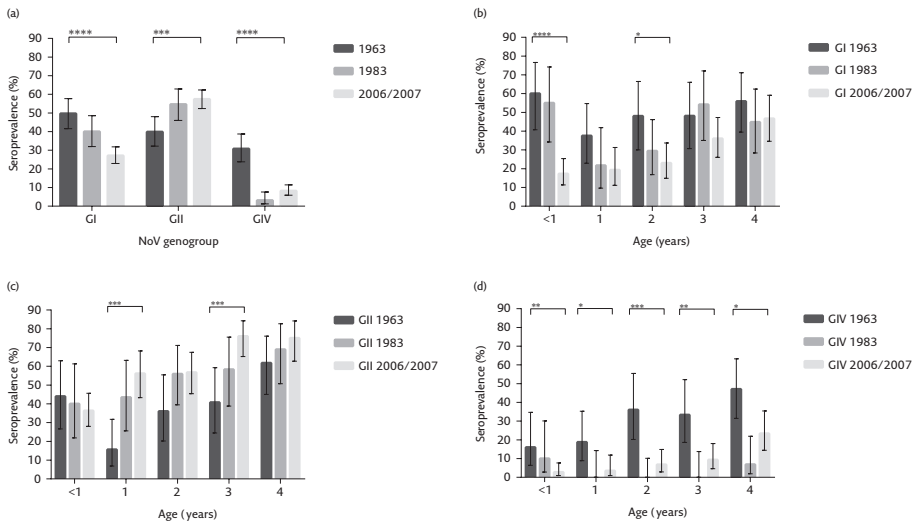
Figure 2 Comparison of norovirus seroprevalence (n=649) stratified to cohort and genogroup (A). Age-related seroprevalence (n = 649) stratified to cohort for norovirus genogroup I (B), genogroup II (C), and genogroup IV (D). Error bars indicate binomial proportion confidence intervals (Wilson score interval). Brackets above bars show significance level as determined by chi-squared test for trend. No bracket = P > 0.05, * = P ≤ 0.05, ** = P ≤ 0.01, *** = P ≤ 0.001, and **** = P ≤ 0.0001.

ranging from 0.01- 0.27. Within a genogroup, some cross-genotype antibody reactivity was observed with correlation coefficients for heterotypic antibody reactivity ranging between 0.52- 0.67. This pattern was not consistent among sera indicating that cross-reactive patterns are complex, reflecting large individual variability rather than a primary technical cause.

B-cell supernatants with single epitope specificity
The observation that the sera of some of the young children were reactive to multiple antigens raised the question whether these children were already infected with multiple norovirus genotypes or contain cross-reactive antibodies, or both. Paired sera of forty children obtained at nine and ten years of age were screened on the protein array for the presence of broadly reacting serum antibody profiles. Two donors with a broad serum profile were selected and B-cells of these donors were polyclonally stimulated to become antibody secreting cells (ASC). Limiting dilution was carried out to create multiple cultures with single epitope specificity. B-cell supernatants were tested on the array to dissect the serological profiles of both donors, using limiting dilution to select for single B cell clones per well[16, 17]. Bulk cultures were first tested and all these cultures reacted with one or multiple antigens, confirming the serological reactivity observed in the sera of these children. For donor 1 only 2 of 14 (14.3%) of pools containing 8000 cells each were positive and both positive clones had single genotype specificity to genotype GII.3 (Table 2). Donor 2 did not meet the clonal criteria as 9 of 16 (56%) pools showed positive reaction to one or multiple norovirus antigens.Some supernatants showed broad GI reactivity, suggesting either the presence of a broadly reactive epitope, or mixed B cell populations. Subsequent testing of individual supernatants pool 12 and 13 confirmed reactivity to multiple genotypes within GI as only 1 of 8 cultures harbour reactivity to norovirus antigens, indicating that these reactivities were caused by a broadly reactive epitope. However, in both donors, multiple clones with single genotype specificity were also obtained, with GII.3 only reactive supernatants in donor 1, and GI.6- and GII.4- specific supernatants in donor 2, showing that technically, the array can measure genotype specific human antibodies.

Seroprevalence
Next the seroprevalence against different norovirus genotypes in children <5 years (n=649) was determined. Based on a pilot experiment with sera of children <1 year of age a cut off for positive samples was set at titre 40 (data not shown). The overall norovirus IgG seroprevalence has not significantly changed over time with 69.2%, 70.0%, and 65.4% of sera positive for at least one genotype in respectively 1963, 1983, and 2006/2007 (Table 3, chi-squared test for trend,

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Table 4 Genotype distribution within positive sera with single antigen response.

Cohort	GI.1		GI.2		GI.6		GI.8		GII.3		GII.4		GII.9		GIV.1		Total	
n / %	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%
1963	2	6.3	10	31.3	5	15.6	2	6.3	1	3.1	0	0.0	4	12.5	8	25.0	32	100
1983	2	6.5	4	12.9	1	3.2	3	9.7	1	3.2	6	19.4	14	45.2	0	0.0	31	100
2006/ 2007	3	3.2	16	17.0	0	0.0	1	1.1	9	9.6	55	58.5	6	6.4	4	4.3	94	100
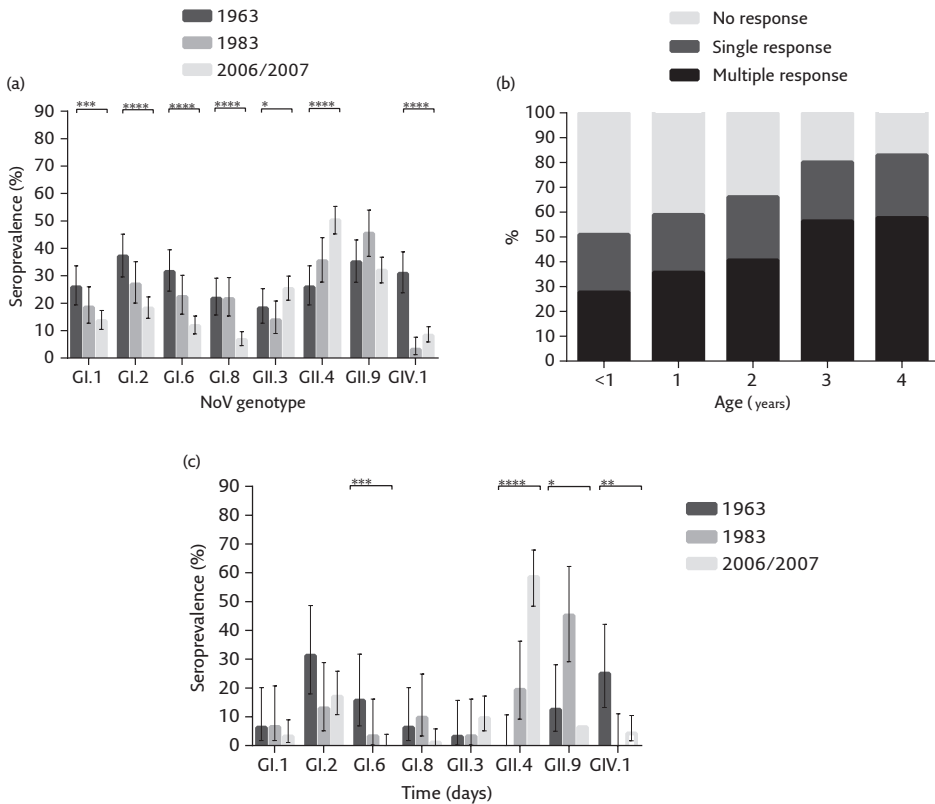
Figure 3 Comparison of norovirus seroprevalence (n=649) stratified to cohort and genotype (A). Age-related broadening of the norovirus immune response (n=649) (B). Comparison of norovirus seroprevalence in sera (n=157) with single response stratified to cohort and genotype (C). Error bars indicate binomial proportion confidence intervals (Wilson score interval). Brackets above bars show significance level as determined by chi-squared test for trend. No bracket = P > 0.05, * = P ≤ 0.05, ** = P ≤ 0.01, *** = P ≤ 0.001, and **** = P ≤ 0.0001.

p-value = 0.3307). In children below one year of age, seroprevalence was already at a high level in 1963 and 1983 (respectively 72.0% and 70.0%), likely reflecting maternal antibodies. In the 2006/2007 cohort the seroprevalence for this age category was significantly lower (42.7%). More than half of the children were already seropositive by the second year of life, increasing to 85.3, 75.9, and 85.0% at age 4 in 1963, 1983, and 2006/2007, respectively. Interestingly, however, substantial differences were observed when breaking the reactivity down to genogroups and genotypes. At the genogroup level, GI seroprevalence dropped over time while the prevalence of GII antibodies increased (Figure 2A). GIV prevalence was much higher in the first cohort, with 30.8% in 1963 compared to 3.1% and 8.2% in 1983 and 2006/2007. Stratified by age, the highest GI seroprevalence decrease was seen among children of age <1 year and 2 years, while the largest GII increase was seen among children of 1 and 3 years (Figure 2B and 2C). Seroprevalence for GIV was significantly higher in all age years in 1963 compared to cohort 1983 and 2006/2007 (Figure 2D). All four tested GI genotypes showed a significant reduction in seroprevalence over time, while the increase in GII antibodies was primarily caused by an increased reactivity to GII.3 and GII.4 antigens (Figure 3A). The antigen with highest seroreactivity was GI.2 in 1963, GII.9 in 1983, and GII.4 in 2006/2007.

Broadening of immune response by increasing age
The multiplex composition of the protein array platform enabled the simultaneous detection of norovirus antibodies to various genotypes and allowed to measure the age-related broadening of the norovirus immune response (Figure 3B). Using a cut off of 40, sera were stratified in three groups: sera without reactivity to any norovirus antigen, sera with reactivity to a single antigen only, and sera with multiple reactivity. A clear age-related increase in sera with multiple responses was observed, while the proportion of sera with monospecific responses remained stable (p-value chi-squared test for trend among respectively multiple, monospecific, and non-responders: <0.0001, 0.6981, <0.0001).

Monospecific responses
To eliminate false positives due to the presence of broadly reactive antibodies, 157 sera from children with antibodies limited to a single antigen were analysed separately (Table 4, Figure 3C). Interestingly, GII.4 antibodies were not detected as unique profile in the 1963 cohort, whereas they constituted 19.4% of monospecific sera in 1983, and were predominant with 58.5% in 2006/2007 indicating a dramatic increase of antibodies to GII.4 or a closely related genotype over time. Furthermore, among antigens tested, GI.2 was predominant in 1963 and GII.9 was predominant in 1983.

Discussion

In this cross-sectional population study the norovirus seroprevalence was investigated among children <5 years of age from which serum was collected in 1963, 1983, or 2006/2007. The results show that norovirus has been a common and widespread infection among Dutch children, at least since 1963, with no indication of an increase in the overall seroprevalence. This confirms prior studies using ELISA or related assays based on recombinant VLPs in China, Finland, India, Japan, Korea, Spain and the United Kingdom, although quite some variation in seroprevalence rates were observed by geographic location and sample year, which may in part be due to a lack of assay standardization[18-22].

It has been hypothesized that the emergence of GII.4 has resulted in a large increase of norovirus outbreaks since 2002[14, 15], and therefore we assessed exposure to individual norovirus genotypes, using a novel protein array. The validation experiments showed that a person's serological profile may contain a mixture of genotype-specific as well as broadly-reactive antibodies with cross genogroup reactivity, although clear discriminatory serological profiles were obtained when looking at post-infection sera. This pattern is in concordance with the higher correlations found between antibodies to antigens of the same genogroup compared to antigens from different genogroups. Based on these observations, we concluded that the study design allows conclusions based on antibody trends by genogroup and genotype, although for the latter the potential for cross-reactive antibodies needs to be considered when interpreting the results.

Our study shows that – despite similar overall seroprevalence – the exposure histories shifted drastically over the period studied, and that the seroprevalence of GII.4 has indeed significantly increased over the last decades, with concomitant reduction in exposure to GI and GIV noroviruses. In theory, the low GII.4 seroprevalence in the 1963 and 1983 cohort could be caused by mismatch between circulating GII.4 variants and the antigen used in our assay (GII.4 US95/96 antigen). This is however unlikely, as sera from patients infected with recent GII.4 variants bound efficiently to the GII.4 US95/96 variant antigen. Therefore, we conclude that the most likely explanation for the observed shift in norovirus exposure is that it reflects true epidemiological changes. The absence of sera from the 1963 cohort that only reacted to GII.4 suggests that either GII4 viruses were not circulating, or that exposure of this genotype occurred at an older age.

Based on these data alone, we cannot draw conclusions on the clinical impact of these shifts in exposure. All norovirus genotypes are associated with diarrheal disease, but GII.4 strains are thought to be more transmissible in healthcare settings, cause more severe disease, and are strongly associated with the seasonal patterns in reporting[23, 24]. The combination of the GII.4 variant pandemics, the association between GII.4 and more severe symptoms, and the high GII.4 infection rates among very young children shown in this study, could potentially explain the increase of norovirus disease reports since 2002. A potential bias in this study is that young children have not yet build up an immune response against norovirus and therefore are likely susceptible to any strain with matching receptor specificity, while adults have already been exposed multiple times to norovirus. Conclusions based on the seroprevalence among young children can therefore not be one-to-one translated to adults.

We also observed broadening of the antibody profile in young children with increasing age, and reactivity to antigens representing genotypes that are rarely found in the Dutch national molecular surveillance. While we cannot rule out that this broadening of antibody profile reflects asymptomatic infections, another explanation is that these effects are caused by high-affinity antibodies directed against shared epitopes on the P domain of VP1, produced by B cells that undergo somatic hyper mutation and clonal selection upon multiple exposures to different genotypes. Although most conserved epitopes are found on the S domain of VP1, the B-cell supernatants in this study and studies with monoclonal antibodies have shown that the P domain contains conserved epitopes as well[25]. An important question is if such antibodies influence the susceptibility to subsequent infection, as has been observed with the discovery of broadly reactive non-hemagglutination inhibition antibodies in influenza A[26].

The shifting of antibody profiles for the different cohorts shows that a GI genotype was dominant in 1963 and a GII other than GII.4 in 1983, which may indicate that multiple dominant genotype switches have taken place before the predominance of GII.4 viruses. Although the overall norovirus seroprevalence did not change over the years in this study, it seems likely that genotype replacements may have concurred with a temporary increase in outbreak activity as is seen with GII.4 variant replacements[27]. Further studies are warranted to measure the effect of changes in the norovirus epidemiology on the norovirus incidence in the population and related costs for the society.

The first norovirus vaccine candidates have been tested in clinical trials and have shown to curb severity of disease after challenge with a homologous strain which may be helpful to reduce hospital admissions in vulnerable patient populations and reduce associated costs[28]. Field efficacy, cross-protection to drifted variants and heterologous genotypes, efficacy in various age groups, and duration of protection needs to be evaluated in future studies. Most importantly, however, our data suggests that special attention needs to be paid to the potential for updating the vaccine composition following changes in the norovirus epidemiology. Current vaccine candidates are based on the norovirus epidemiology with dominance of GII.4, but with the recent emergence of GII.17 in Asia the vaccine candidates may need to be updated[29]. Continued surveillance and a better understanding of norovirus epidemiology is essential knowledge for an optimal vaccine design.

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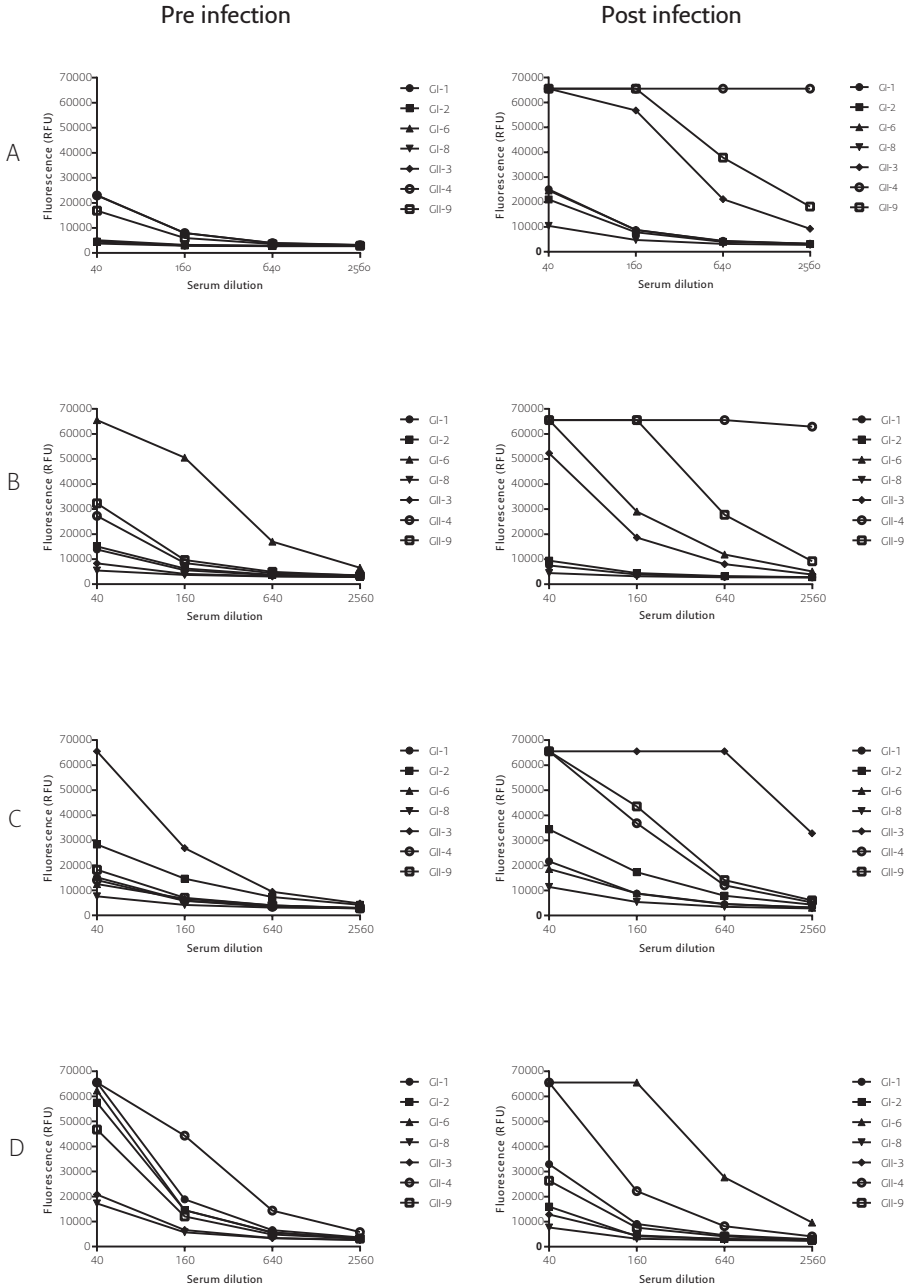
Supplemental Figure S1 Paired sera of four norovirus RT-PCR confirmed patients (ages 5, 47, 17, and 12 y respectively) infected with GII.4 Den Haag 2006b (A), GII.4 New Orleans 2009 (B), GII.3 (C), or GI.6 (D) tested against 7 different norovirus P particles on the protein array platform.
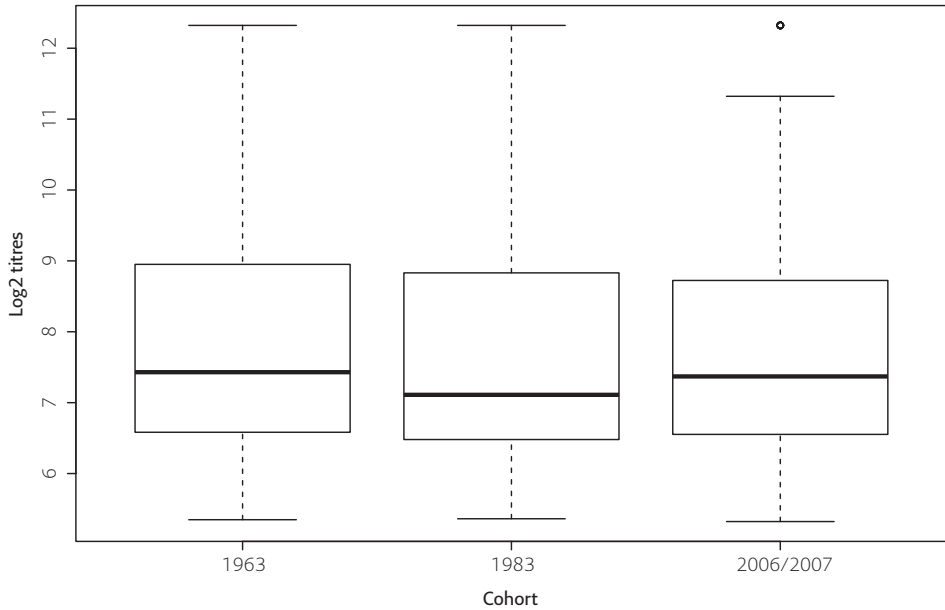
Supplemental Figure S2 Distribution of positive titres against any antigen among cohorts

Supplementary Table S1 Recombinant norovirus antigens used to test sera on the protein array platform

Genotype	Antigen type	Accession number
GI.1	P particle	M87661
GI.2	P particle	AF435807
GI.6	P particle	AF538678
GI.8	P particle	AF538679
GII.3	P particle	U22498
GII.4	P particle	AY038600
GII.9	P particle	AY038599
GIV.1	VLP	AF414427

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Chronic norovirus infection among solid organ recipients in a tertiary care hospital, the Netherlands, 2006 – 2014

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Abstract

Immunocompromised patients can suffer prolonged norovirus symptoms and virus shedding for many years. Little is known about the prevalence of chronic norovirus infection among solid organ transplant (SOT) recipients. In this study, 2182 SOT recipients were retrospectively tested for chronic norovirus infection. The first and last norovirus positive faecal samples of SOT recipients were sequenced to distinguish between persisting infection and re-infection. Patient charts were reviewed to obtain data on health status and treatments.
In all, 101 of 2182 (4.6%) recipients were norovirus infected and 23 (22.8%) of these developed chronic norovirus infection. Chronic norovirus infection was found among allogeneic heart, kidney and lung transplant recipients. The median shedding period at the end of the study period was 218 days (range 32-1164 days). This study shows that chronic norovirus infection is not a rare phenomenon among SOT recipients in a tertiary-care hospital. Further research is needed to study the risk of norovirus transmission to other immunocompromised patients in the hospital and to the general population.

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Introduction

Noroviruses belong to the *Caliciviridae* and are the predominant viral cause of acute gastroenteritis in health-care institutions and community settings worldwide[1]. Noroviruses are a genetically and antigenically highly diverse group of positive stranded RNA viruses which can be subdivided in at least six genogroups (G) of which GI, GII, and GIV are known to infect humans[2]. Clinical symptoms of infected persons include watery diarrhea, stomach pain, vomiting, nausea, weight loss, as well as headache and fever. Norovirus outbreaks occur all year round but the frequency of outbreaks peaks in the winter season in temperate northern hemisphere countries. Norovirus is highly contagious and easily transmitted via person-to-person contact, contaminated surfaces, or via contaminated food or water[3].

In immunocompetent individuals, symptoms are self-limiting and usually resolve within 2-3 days[4]. Virions are shed in highest quantities during the acute phase of infection and shedding continues after symptoms have disappeared. Human challenge studies have shown that the median viral shedding in healthy adults is 28 days (range 13-56 days)[5]. In recent years, anecdotal reports have described norovirus as a causative agent of persisting infections in the immuno-compromised host, including solid organ transplant recipients, patients with congenital immunodeficiency, patients treated with chemotherapy, and patients infected with human immunodeficiency virus (HIV)[6-10]. These patients can suffer from prolonged symptoms that may last multiple years leading to severe dehydration, malnutrition, patient discomfort, acute renal failure, and eventually death[11, 12]. Norovirus has parallels to hepatitis E virus that originally was thought to cause solely acute infection and for which recently has been shown to cause chronic infection among SOT recipients[13]. Patients with a chronic norovirus infection pose a potential public health risk since they shed virus in high quantities, may act as a reservoir for nosocomial transmission, and may be reservoirs for the development and emergence of new antigenic variants in the general population[14-16].

Improved immunosuppressive drugs have significantly reduced the incidence of organ rejections among SOT recipients and drastically improved patients' survival with the consequence of a higher susceptibility to reactivation of latent infections, and hospital and community acquired infections[17, 18]. The higher infection rates among SOT recipients might be caused by the improved survival time[19]. The effect of immune suppression on the incidence of chronic norovirus infection has not been studied comprehensively, as information is limited to case reports and small series[20]. We hypothesize that chronic norovirus

infection is more common among SOT recipients than currently recognized. This retrospective cohort study aims to systematically estimate the incidence of chronic norovirus infection among patients with solid organ transplantations in a tertiary care hospital and to get better insight in the patient population susceptible to chronic norovirus infection.

Materials and methods

Study population

The Erasmus Medical Center (EMC) and affiliated hospitals comprise a 1332-bed university medical centre. During the study period between January 2006 until December 2014, 2182 patients received at least one lung, liver, kidney, or heart transplant.

Data and sample collection

We retrospectively retrieved sex, date of birth, date of transplantation, date of hospital admission, and type of transplanted organ of all SOT patients during the study period from the hospital patient database. Next, we retrieved norovirus RT-PCR results of SOT patients who had a faecal sample routinely tested from the laboratory database. The collection of anonymized data and biobanked specimens was approved by the Erasmus MC ethical committee (MEC-2015-053). Norovirus RT-PCR was routinely implemented for diagnostic purposes in 2006 and used throughout the years. Recipients with at least one norovirus RNA positive sample post transplantation were assigned as norovirus infected. The first and last available faecal samples of patients with minimal two norovirus positive samples and minimal time between sampling moments of one month were retrieved from the hospital biobank, and sequenced to determine the norovirus genotype and to distinguish between chronic infections and reinfections. All faecal samples used in this study had been sent to the hospital laboratory for diagnostic purposes and stored at -80° C until further use. A database search was performed to obtain *Clostridium difficile* diagnostic requests of norovirus positive patients to get a better understanding of potential missed norovirus positive samples. We assumed that patients with a *C. diff.* diagnostic request (test negative) have symptoms of gastroenteritis. Therefore, patients with norovirus diagnosis without follow up, but who were repeatedly tested for *C. diff.* might reflect missed chronic norovirus patients. The samples submitted for *C. diff.* were not stored in the EMC biobank and could therefore not be tested for norovirus. Dutch norovirus surveillance data was retrieved from the noronet database (www.noronet.nl).

Virus detection
Norovirus was detected with a routine semi quantitative real time Polymerase Chain Reaction (RT-PCR) assay as described[21]. Briefly, a faeces suspension was prepared with 100 mg or 200 μl faecal sample in 600 μl STAR buffer (Roche Diagnostics, Basel, Switzerland) and 80 μl chloroform. After centrifugation, 190 μl of supernatant, 250 μl of lysis buffer (Roche Diagnostics) and 10 μl of an internal extraction control (phocine distemper virus) were transferred to a MagnaPureLC (Roche Diagnostics) for total nucleic acid (TNA) isolation. 20 μl viral RNA was amplified using the TaqMan Fast Virus 1-Step Master Mix (Life Technologies, Carlsbad, California, United States), primers, and uracil-N-glycosylase (Life Technologies) in a final volume of 50 μl according to manufacturer's instructions. The cycle threshold (Ct) value of both, the positive control sample and the internal extraction control, should be within the range of three standard deviations from the mean to approve a PCR run.

Virus sequencing
A newly developed protocol was used to sequence a fragment of approximately ~1000 bp overlapping ORF1 and ORF2 thus enabling genotyping of both the polymerase and capsid gene with a single protocol as recommended by Kroneman *et. al.* 2013[22]. After RNA extraction by MagNA Pure 96 (Roche Diagnostics), 5 μl viral RNA was reverse transcribed into cDNA in a total volume of 10 μl using 2.5 μM primer A (Supplementary Table 1), 1x RT-Buffer, 0.1 mM dNTPs, 5 mM DTT, 20 units Rnase Out, and 100 units SuperScript III enzyme (Thermo Fisher, Waltham, USA) at subsequently 22° C for 10 min, 50° C for 1 hour, and 95° C for 5 min. An outer PCR reaction was performed on 2.5 μl cDNA in a total reaction volume of 25 μl using 1x HotStarTaq mastermix (Qiagen, Hilden, Germany), 1 μM primer A and 1 μM primer B for genogroup I (GI) viruses and 1 μM primer A and 1 μM primer C for GII viruses at 15 min 95° C, 30 cycles 94° C 30 s, 42° C 30 s, 72° C 90 s, followed by 72° C for 300 s. A nested PCR was performed on 1.5 μl PCR products in a total reaction volume of 25 μl using 1x HotStarTaq mastermix and 1 μM primer D and E for GI, or 1 μM primer F and G for GII at 95° C 15 min, 4 cycles 94° C 30s, 55° C 30s, 72° C 90s, followed by 32 cycles 94° C 30s, 60° C 30s, 72° C 90s, and subsequently 72° C for 90s. PCR products were subsequently sequenced using ABI Prism BigDye Terminator v3.0 ready reaction cycle kit (Applied Biosystems, Bleiswijk, the Netherlands) using primers H-K.

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Data analysis and clinical definitions
A chronic patient was defined as a recipient with at least two norovirus RT-PCR positive faecal samples, positive for ≥30 days, with an identical ORF1 and ORF2 genotype in the first and last sample, and clustering of both samples in a phylogenetic tree. Patients with at least two norovirus RT-PCR positive faecal samples, positive for ≥30 days, but not with an identical ORF1 and ORF2 genotype or clustering of both samples in a phylogenetic tree were attributed as re-infected. Sequence trace files were analyzed using BioNumerics v7.5 (Sint-Martens-Latem, Belgium) and genotyped by the norovirus genotyping tool[23]. Phylogenetic analysis was done based on maximum likelihood analysis and the Kimura 2-parameter model and 1000 bootstrap replicates using MEGA v6.0[24]. The Kimura 2-parameter model showed to be the best-fit substitution model as determined by the model selection module of MEGA v6.0. Norovirus sequences were submitted to Genbank (accession numbers: KX446494 - KX446537).

Results
A total of 2182 SOT recipients were included in the study: 174 heart- (HTx) of whom 10 were <10 years of age, 106 lung- (LungTx), 378 liver- (LiverTx), 1510 kidney- (NTx) of whom 39 were <10 years of age, and 14 multiple organ transplant recipients (Table 12). The treating clinician decided to obtain a sample for norovirus diagnosis post transplantation based on the clinical presentation of the patient for 546 of 2182 (25.0%) recipients. One hundred one of 2182 SOT recipients (4.6%) tested positive for norovirus by RT-PCR at least once post transplantation (Figure 1A and Table 1). Thirty SOT recipients with norovirus (29.7%) were sampled more frequently with a minimal collection span of one month, and chronic infection could be confirmed by sequence analysis in 23 of these (76.7%) (Supplementary Figure 1). Three persons (10.0%) were re-infected, and for 4 of 30 SOT recipients (13.3%) samples were not available or could not be sequenced due to low viral load. Chronic norovirus infection was identified among NTx (recipients <10 years of age n=5, ≥10 years of age n=15), LungTx (n=2), HTx (n=1, recipient <10 years of age), and not among LiverTX or multiple SOT recipients.

Initial norovirus diagnostic testing was requested for unexplained diarrhea for 21 of 23 (91.3%) recipients and for 2 of 23 (8.7%) this data could not been retrieved from the hospital medical records (Table 2). Symptoms of diarrhea or normal stool alternated with diarrhea were reported for 14 of 23 (60.9%) recipients during the course of the chronic period for at least 2 weeks post initial sampling. For two (8.7%) patients feeding disorder (n=1) and constipation (n=1)

(a)

(b)
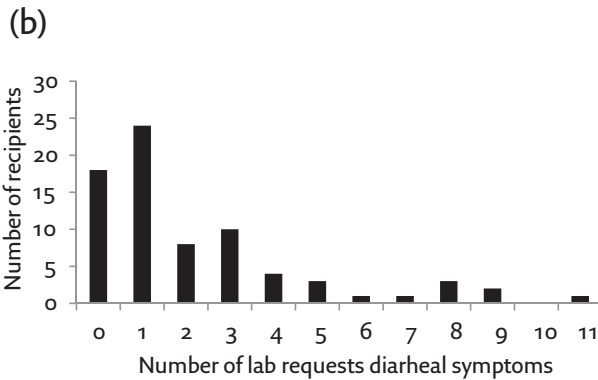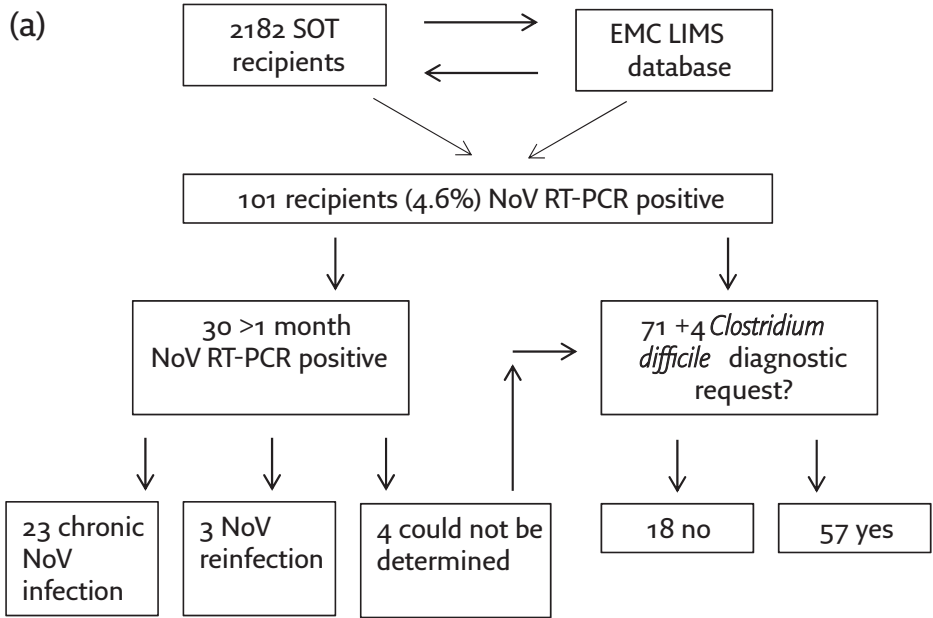
Figure 1 (A) Study diagram, EMC = Erasmus Medical Center, NoV = norovirus. (B) Number of (test negative) diagnostic requests for *C. diff.* indicative for diarrheal symptoms among norovirus positive SOT recipients that had only been tested once (n = 75).

Table 1 Overview of norovirus infections among SOT recipients, 2006-2014.

Transplant	Recipients	RT-PCR confirmed NoV infection		Confirmed chronic NoV infection	
	n	n	%	n	%
Heart	174	9	5.17	1	0.57
Liver	378	5	1.32	0	0.00
Lung	106	9	8.49	2	1.89
Kidney	1510	77	5.10	20	1.32
Multiple*	14	1	7.14	0	0.00
Total	2182	101	4.62	23	1.05

*Multiple transplants: ten liver and kidney, two lung and kidney, and two heart and kidney.

were reported during follow-up which might be related to norovirus infection. Six recipients (26.1%) did not report norovirus related symptoms during the course of the chronic NoV episode and data was missing for the remaining recipient (4.3%). The median viral shedding period at the end of the study period for chronic patients lasted a median of 218 days (range 32-1164) (see Table 2 and supplementary Figure 2). 16 of 23 (69.6%) SOT recipients with sequence confirmed chronic norovirus infection shed norovirus for more than 90 days. The median time span between transplantation and initial sampling was 301 days (range: 26-7387). All 23 chronically infected SOT recipients had been treated with mycophenolate mofetil (MMF) as initial immunosuppressive therapy in combination with tacrolimus or sirolimus, and 20 of 23 (87.0%) recipients were using corticosteroids. Thirteen of 23 (56.5%) of recipients needed a major change in the immunosuppressive therapy regime during the norovirus chronic period.

As norovirus testing is not always done as part of the routine evaluation of diarrhea in transplant recipients, we reviewed the diagnostic requests for *C. diff.*, as a proxy for being symptomatic. In the month before initial norovirus detection, stool samples of 9 of 23 (30.4%) chronic norovirus cases had been sent to the laboratory for *C. diff.* testing, and in the month after the last norovirus positive stool test, this was done for 2 of 23 (8.7%) chronically infected recipients (Supplementary Figure 2). To get a better understanding of potential missed chronically infected norovirus recipients, we analysed the number of diagnostic requests for *C. diff.* with negative test outcomes, for the norovirus positive SOT recipients that had only been tested once (n=75,

Figure 1A). Repeated *C. diff.* testing had been requested for 33 of 75 (44.0%) of these patients, indicative for prolonged periods of unexplained symptoms and potentially missed chronic norovirus cases (Figure 1B).

The virus diversity observed in the chronic patients was similar to that observed in the national surveillance data (Supplementary Figure 2). Only recipient number 3 and 16 were infected by a GII.4 variant that was not widely present at that moment in the Netherlands. Surprisingly, recipient 3 was infected by GII.P4 New Orleans 2009-GII.4 New Orleans 2009 early 2008, almost a year before this variant was first detected in the surveillance. Three recipients had repeated infections: they switched from genotype GII.P4-GII.4 to GI.P7-GI.7, GII.P4-GII.4 to GII.Pe-GII.4, and GII.P4-GII.4 to GI.Pb-ORF2 unknown (this could not be sequenced due to a low viral load, data not shown).

Discussion

This study has examined the incidence of (chronic) norovirus infection among the total population of solid organ transplant recipients in a tertiary care hospital. We found 4.6% of SOT recipients to be norovirus infected. Chronic norovirus infection was found among 23 of 2182 (1.1%) SOT recipients and shows that chronic norovirus infection is not a rare phenomenon. This study more than doubles the number of chronic norovirus cases described in the literature (Supplementary Table 2). Strengths of this study are the size of the patient cohort, and the confirmation of chronic infection by genome sequencing.

Among SOT patients, chronic norovirus infection has been reported in literature among pancreas, renal, heart, liver, intestine transplant recipients, and among multi solid organ or a combination of solid organ and stem cell transplant recipients (Supplementary Table 2). Other studies reported chronic diarrhea related to norovirus among SOT recipients, but without distinction between persisting and re-occurring infections through sequencing[25-27].

This study had a retrospective setup and relied on the decision of a clinician to request for norovirus diagnostics. A limitation of the study is that the reported incidence rate therefore should be interpreted as a lower limit and presumably more chronically infected patients and longer shedding periods would have been identified with more frequent and systematic sampling, as was suggested by the more frequent (test negative) diagnostic requests for *C. diff.*. Furthermore, an unknown proportion of recipients change hospital over time, which potentially caused an underestimate of the prevalence as well.

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Table 2 Characteristics of SOT recipients with confirmed chronic norovirus infection

	Confirmed recipients (n=23)
Characteristic	
Median age in years (range)	53 (2-72)
HTx recipients <10 years of age	1 (4.3%)
NTx recipients <10 years of age	5 (21.7%)
LungTx ≥10 years of age	2 (8.7%)
NTx recipients ≥10 years of age	15 (65.2%)
Number of females (males)	9 (14)
Median days between Tx and first NoV positive sample (range)	301 (26 -7387)
Median days viral shedding sequence confirmed (range)	218 (32-1164)
Initial diarrhea symptoms	21 (91.3%)
Initial symptoms not recorded	2 (8.7%)
Tested norovirus negative post chronic period	2 (8.7%)
Mortality (not related to norovirus infection)	3 (13.0%)
Initial immunosuppressive therapy	
Mycophenolate mofetil	23 (100%)
Tacrolimus	22 (95.7%)
Sirolimus	1 (4.3%)
Basiliximab	1 (4.3%)
Corticosteroids	20 (87.0%)
Follow-up	
Symptoms > 2 weeks after initial diagnosis	
Diarrhea symptoms	7 (30.4%)
Occasional diarrhea symptoms	7 (30.4%)
Feeding disorder	1 (4.3%)
Constipation	1 (4.3%)
No complaints reported	6 (26.1%)
No data about symptoms available	1 (4.3%)
Immunosuppression	
Major changes immunosuppression due to diarrhea symptoms	13 (56.5%)
Reason change immunosuppression not reported	5 (21.7%)
No changes immunosuppression	5 (21.7%)

All recipients with chronic norovirus infection and available medical record information initially showed prolonged symptoms of diarrhea while during follow-up some patients reported continuous symptoms of diarrhea and others only diarrhea alternated with normal stool samples or even absence of norovirus related symptoms. This mixed presentation shows that diagnosis of norovirus infection in organ transplant patients based on clinical symptoms is complex. Diarrhea is a common complication that can be caused by treatment, Graft versus Host Disease (GvHD), multiple viral, parasitic, or bacterial infections, or a combination of these[28]. Since different aetiologies require fundamentally different treatment regimens, reliable diagnostic assays are an essential tool to distinguish norovirus infection from other causes of gastroenteritis[29]. Early recognition of norovirus infection among immunocompromised patients may help to fine-tune the immunosuppressive therapy, prevent uncomfortable medical examination like colonoscopy and biopsies, prevent norovirus transmission, improve the quality of life of patients, and reduce healthcare costs[30].

Despite the immunocompromised state leading to chronic infection, 3 SOT recipients apparently cleared the infection, but were re-infected. It is conceivable that the level of immunosuppression fluctuates, as this is tuned by the treating physician based on evaluation of engraftment. Unfortunately, we could not retrieve this information from the clinical records to address this question with certainty. Two patients were re-infected with a virus belonging to another genogroup, which is what would be predicted as within genogroup protective immunity of short duration which has been shown earlier in naturally infected healthy individuals in a one year cohort study from Cameroon[31].

In conclusion: chronic norovirus infection is not uncommon among SOT patients and studies are needed to get a better understanding of the patient populations at risk and clinical consequences for chronic norovirus infection, to study the risk of norovirus transmission to other immunocompromised patients and the general population, and to development an antiviral therapy for the treatment of norovirus infection. Hospitals need to consider improvement on early recognition of chronic norovirus infection and infection control measures to prevent transmission to the vulnerable immunocompromised patient population.

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Acknowledgement

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Supplementary Table 1 Primers designed for norovirus GI, GII, and GIV RNA genome amplification and sequencing.

Code	Genogroup	Sequence	Use
A	GI and GII	AIYTTICCIGCIGWRAAIGCRTT	Reverse transcription / amplification
B	GI	ATGAAYACAATNGARGAYGGNCC	Amplification
C	GII	ATGAAYATGAAYGARGAYGGNCC	Amplification
D	GI	GACTACAGCTTGGGAYTCNACNCAR	Amplification
E	GI	ACTCTCATATTCCCAACCCANCCRTTRTACAT	Amplification
F	GII	GACTACTCTCGGTGGGAYTCNACNCAR	Amplification
G	GII	ACCTCAAAACCACCTGCATANCCRTTRTACAT	Amplification
H	GI	ACTCTCATATTCCCAACCC	Sequencing
I	GI	GACTACACAGCTTGGGA	Sequencing
J	GII	ACCTCAAAACCACCTGCAT	Sequencing
K	GII	GACTACTCTCGGTGGGA	Sequencing

Supplementary Table 2 Molecular evidence for chronic norovirus infection among SOT patients as described in literature

First author	Transplant	Genotype	(RT)-PCR confirmed patients	Patients confirmed by sequence analysis
Nilsson[1]	Heart	GII	1	1
Kaufman[2]	Intestine	GII.P4	1	0
Florescu[3]	Small bowel	NR	1	0
Lee[4]	Combined liver, pancreas, and small bowel	NR	1	0
Westhoff[5]	Kidney	2 GII	2	0
Schorn[6]	Kidney	4 GII.P4, 3 GII. P4-GII.4, GII.P7, GII.17	9	9
Ebdrup[7]	Heart	NR	1	0
Boillat-Blanco[8]	Lung combined with hematopoietic stem cell Tx	GII.4*	1	1
Roos-Weil[9]	Kidney	8 GII.4, GII.2, 2 GII.6, GI.3, 3 GII**	15	0
Engelen[10]	Heart	NR	1	0
Hoffman[11]	Small bowel	GII.4	1	1
Chagla[12]	Combined Kidney and Pancreas	GII.4**	1	1
Echenique[13]	Pancreas	GII.4	1	1
Subtotal			**36**	**14**
This study	Kidney, Lung, Heart	14 GII.P4-GII.4, 3 GII.Pe-GII.4, 2 GII.P21-GII.3, 2 GII.P7-GII.6, GII.P7-GII.7, GII. P2-GII.2	30	23
Total			**66**	**37**

*Infection started before lung transplantation

**This study did not report whether genotypes are indicated for ORF1 or ORF2

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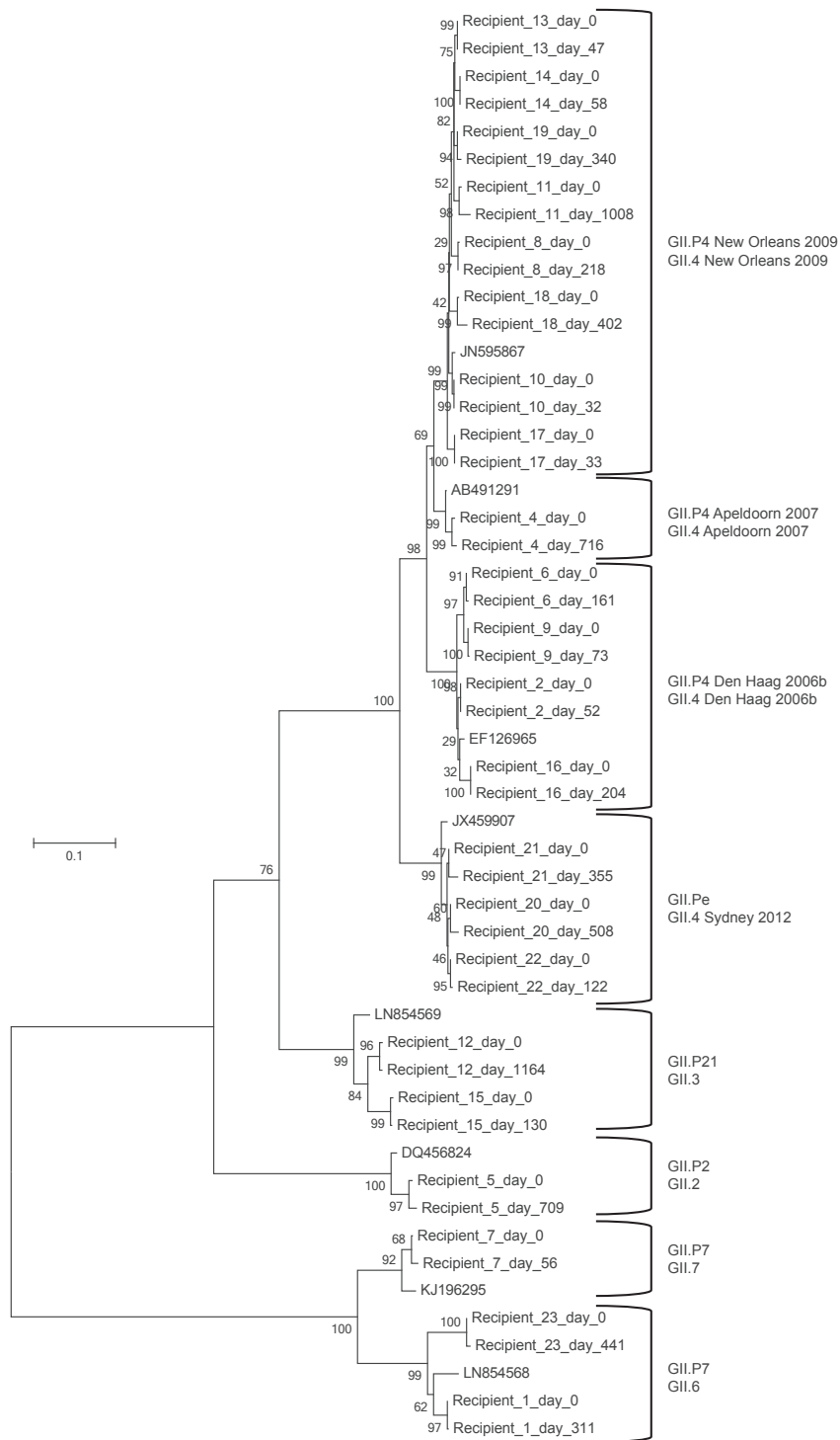
Supplementary Figure 1

Supplementary Figure 1 Maximum likelihood phylogenetic analysis of partial polymerase and partial capsid norovirus sequences (938 bp) collected from the first and last available sample of n=22 SOT patients using the Kimura 2-parameter model for nucleotide substitution and 1000 bootstrap replications. Indicated days are days relative to the sample date of the first positive sample for each individual patient. Patient number 3 (GII.P4 New Orleans 2009 - GII.4 New Orleans 2009, 548 days RT-PCR positive) was excluded from the figure

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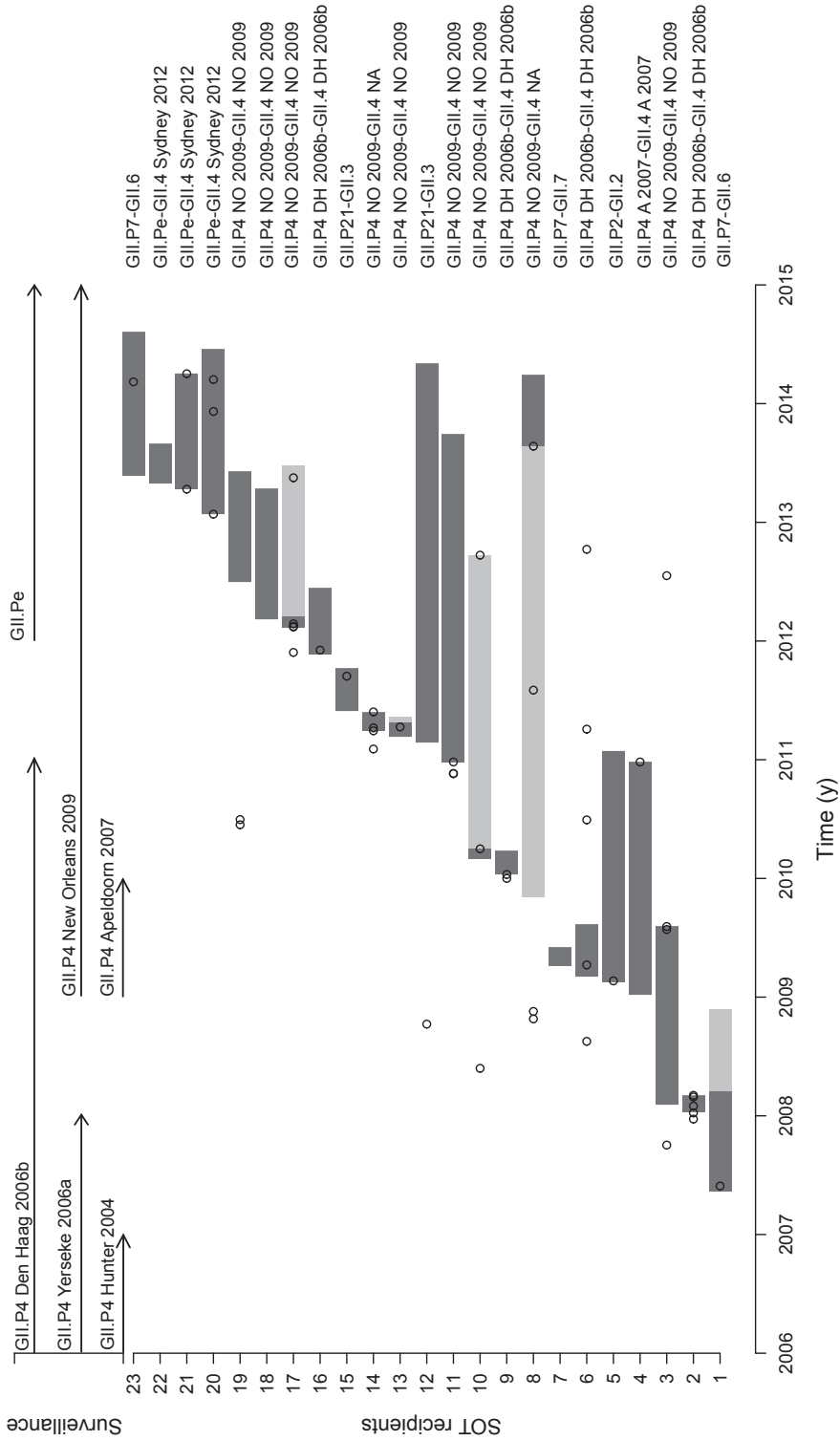
Supplementary Figure 2

Supplementary Figure 2 Time line chronically norovirus infected SOT recipients (n = 23) and comparison to Dutch national surveillance norovirus GII.4 variants. Dark grey boxes indicate period of sequence confirmed chronic infection. Light grey box indicate norovirus RT-PCR positive period for patients with samples that could not be sequenced due to limited availability or low viral load. Circle symbols indicate diagnostic requests for C. diff. as an indication for symptoms of gastroenteritis and potential missed norovirus diagnosis. The arrows indicate years for which the contribution of GII.4 variants was more than 10% of all GII.4 variants. GII.Pe is shown in the graph since this polymerase genotype recombines frequently with the capsid of the GII.4 Sydney 2012 variant. GII.4 variant abbreviations: NO = New Orleans, DH = Den Haag, NA = Not Assessed, A = Apeldoorn.

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Whole genome next-generation sequencing to study within-host evolution of norovirus (NoV) among immunocompromised patients with chronic NoV infection

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Abstract

The genus *Norovirus* comprises large genetic diversity and new GII.4 variants emerge every 2-3 years. It is unknown in which host these new variants originate. Here we study whether prolonged shedders within the immunocompromised population could be a reservoir for newly emerging strains. Faecal samples (n=65) from immunocompromised patients (n=16) were retrospectively selected. Isolated viral RNA was enriched by hybridization with a custom norovirus whole-genome RNA bait set and deep sequenced on the Illumina MiSeq platform. Patients shed virus for average 352 days (range: 76–716 days). Phylogenetic analysis showed distinct GII.4 variants in 3 out of 13 (23%) patients. The viral mutation rates were variable between patients, but did not differ between various immune status groups. All within host GII.4 viral populations showed amino acid changes at blocking epitopes over time and the majority of VP1 amino acid mutations were located at the capsid surface. This study found viruses in the immunocompromised host that are genetically distinct from viruses circulating in the general population and these patients therefore may contain a reservoir for newly emerging strains. Future studies need to determine whether these new strains are of risk for other immunocompromised patients and the general population.

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Introduction

Noroviruses are rapidly evolving positive stranded RNA viruses and are a predominant non-bacterial cause of acute gastroenteritis in all age groups worldwide[1]. The genus *Norovirus* demonstrates large genetic diversity and is divided into at least seven genogroups and subdivided into >30 genotypes[2]. Noroviruses evolve through recombination and through accumulation of mutations leading to changes in epitopes targeted by protective antibodies (antigenic drift). Antigenic drift has been observed for several genotypes but is most prominent in the predominant lineage genogroup II, genotype 4 (GII.4), of which a new antigenic variant arises and typically replaces the previously established variant every 2-3 years[3]. In the last two decades, six distinct GII.4 variants emerged with global spread: US 1995/96, Farmington Hills 2002, Hunter 2004, Den Haag 2006b, New Orleans 2009, and Sydney 2012[4]. The evolution of norovirus GII.4 follows a stepwise or epochal pattern with limited genetic diversity within variants and large genetic distance, up to 25 amino acid (aa) mutations in the 541 (4,6%) aa VP1 protein, between variants[5]. Most genetic variation occurs in the protruding (P) domain of the VP1 protein, which is exposed to the outer surface of the capsid and shown to contain blockade epitopes and the receptor binding pocket[6]. The emergence of epidemic GII.4 variants has been associated with a significant increase in the number of outbreaks leading to an increase in morbidities and mortality in risk groups including children, elderly, and immune compromised patients, and related costs for society[7-9].

Intermediate strains that fill the genetic gap between successive GII.4 variants are rarely detected which raise questions on how and where these strains emerge. Noroviruses circulating in animal reservoirs have been suggested as a source of norovirus variation. Although some animal genotypes are relatively closely related to human genotypes, GII.4 strains are rarely found in animals, making zoonotic introduction an unlikely hypothesis for the observed pattern of evolution of GII.4[10, 11]. Therefore, it is tempting to suggest that norovirus GII.4 diversity originates within the human population. The immune compromised host is a potential reservoir for norovirus variants[12]. While norovirus infection is self-limiting in immuno-competent hosts, it is increasingly recognised that immune compromised individuals can suffer prolonged symptoms and can shed noroviruses for long periods of time, up to years. The failure to clear the virus coupled with high viral loads may result in substantial sequence diversity within a single chronic patient[12], but an important question is whether chronic norovirus shedders have sufficient (mucosal) immunity to drive selection of antigenic variants. Incidental evidence suggests changes in viruses over time

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in these patients, including aa changes in B-cell epitopes, but only a limited number of patients were assessed[13-15].

In this study, we asked if patients with chronic norovirus infection (chronic shedders) could be a reservoir for genetically distinct variants. A selection of stool samples collected from 16 patients during a time span of 6.5 years was deep sequenced to analyse norovirus evolution within patients over time.

Methods
Study population and sample selection
Faecal samples (n=65) of immune compromised patients (n=16) were retrospectively selected from the Erasmus Medical Center biobank and screened for chronic infections. Solid organ transplant (SOT) patients with chronic norovirus infection were derived from an earlier study on the prevalence of chronic norovirus infection in a tertiary care hospital and additional screening was performed for non-SOT immune compromised patients[16]. For each patient, we selected the first and last known norovirus positive sample as a minimum, as well as intermediary samples, depending on availability. All samples had been obtained for diagnostic purposes and tested positive for norovirus RNA by an in-house standard diagnostic RT-PCR assay with Ct<32. Samples were stored at -80°C freezer. The Erasmus MC ethical committee approved the study under registration number MEC-2015-025.

Viral RNA isolation
A clarified 10% (w/v) faecal suspension was prepared in PBS and viral RNA was extracted from 140 µl of the suspension using the Qiamp viral RNA mini kit (Qiagen, Hilden, Germany). The RNA extract (40 µl) was concentrated using a vacuum concentrator Savant Speed to 11 µl and used for first strand cDNA synthesis, using random hexamers and Superscript III Reverse Transcriptase (Thermo Fisher Scientific, Waltham, MA). The resulting cDNA was used for double stranded DNA synthesis using NEBNext mRNA Second Strand Synthesis Module (NEB, Ipswich, MA) according to the manufacturer's instructions. Double stranded DNA was purified and concentrated with Genomic DNA clean and concentrator kit (Zymo research, Irvine, CA) with a final 30 µl elution. The DNA yield was quantified using a Qubit® 2.0 Fluorometer (Invitrogen, Carlsbad, CA).

SureSelect target enrichment and library preparation for next generation sequencing
Overlapping 120-mer RNA baits complementary to and spanning partial or complete reference genomes of 987 norovirus strains were designed by the

PATHSEEK consortium[17]. The specificity of the baits was verified by nucleotide (nt) BLAST search against the NCBI Human Genomic Plus Transcript database. The custom designed norovirus bait library was uploaded to E-array and synthesised by Agilent Technologies. Norovirus cDNA samples, supplemented optionally with carrier G147 Human genomic DNA (Promega) to accomplish a total of 200 ng DNA, were sheared for 120 seconds using a Covaris E210 (duty cycle 5%, Peak Incident Power 4 and 100m cycles per burst). End repair, non-templated addition of 3' adapter ligation, hybridisation, enrichment PCR and all post-reaction clean-up steps were performed according to the SureSelect Illumina Paired-End Sequencing Library XT protocol[17]. All recommended quality steps were performed between steps.

Illumina sequencing
Libraries were multiplexed at 48 sample libraries per run. Paired end 300nt sequencing was performed on an Illumina MiSeq sequencer using the 600 cycle v3 reaction kit. Base calling and sample demultiplexing were performed using the default settings on the MiSeq, generating paired FASTQ files for each sample.

De novo assembly and identification of viral genomes.
Raw sequencing reads were processed to remove adapters and trimmed from the 3' end to reach median Phred score >=35 using QUASR[18]. The reads were assembled into contigs using *de novo* assembly with SPAdes 3.9.0[19]. Calicivirus-encoding contigs were identified with a modified SLIM algorithm combined with ublast[20, 21]. Partial but overlapping contigs were joined into full-length genome sequences using Geneious v9.0.4 (Biomatters Ltd) and ambiguities were resolved by directly counting 21 nt motifs containing the ambiguous site in the quality-controlled short read data. The python script used to check ambiguous sites is available at Github (https://github.com/mlcotten). Nt and aa uncorrected maximum pairwise distance between consensus sequences were calculated using MEGA v7.0.18[22]. Norovirus consensus sequences were submitted to GenBank (accession numbers: MF140633 - MF140697).

Minimum-spanning network and Maximum-likelihood phylogenetic trees
All *de novo* assembled genomic sequences were aligned using AliView v1.16[23]. To investigate the clustering patterns, a minimum-spanning network within and between patients was constructed in PopART v1.7 (http://popart.otago.ac.nz/index.shtml) with an epsilon of zero[24]. Maximum-likelihood phylogenetic trees were constructed in RAxML under GTR-Γ model of substitutions with 500 bootstraps[25]. USEARCH version 7.0 was used to select representative GII.4

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reference background sequences using a similarity cut-off of 98.6%[20]. The number of available GenBank reference sequences for GII.3, GII.6 and GII.7 was limited and therefore did not need to be reduced.

Minor variant analysis
For each sample, the quality-controlled short reads were mapped to the assembled genome using BWA[26]. The resulting pileup file was parsed to identify positions with non-consensus nt. Only positions with a minimum coverage of 100 reads and only reads with minimum quality score of 35 were reported. Positions with >10% minor variant frequency were collected and graphed by genome position. While this threshold is relatively high, it was set to avoid over-interpretation of the data in view of contamination problems that have been reported for illumine platform based NGS (https://www.illumina. com/content/dam/illumina-marketing/documents/products/whitepapers/ index-hopping-white-paper-770-2017-004.pdf?linkId=36607862). Our future work aims to optimize lower frequency variant calling based on NGS deep sequencing applications for clinical decision making. The python scripts used for the analysis and graphing are available at Github (https://github.com/mlcotten).

Results
Patient profiling
Patients were sampled between 2008 and 2014, had an average age of 46 years (range: 3-72 years), and were infected with norovirus GII.P4–GII.4, GII. Pe–GII.4, GII.P7–GII.6, GII.P7–GII.7, or GII.P21–GII.3 (Table 1). Patients were immune compromised due to solid organ transplantation (kidney n=8, lung n=2), allogeneic stem cell transplantation (n=2), leukaemia (n=2), or immuno-logical disorders (n=2). The average norovirus shedding time (at the end of the study period) was 352 days (range: 76 – 716 days).

Quality control
For initial quality assessment, we compared the total number of reads, number of norovirus specific reads, and genome read depth for each sample in relation to the norovirus quantitative RT-PCR cycle threshold value (Supplementary Figure 1 panels A, B, and C). The total number of short reads per sample showed a median value of 311558 per sample, with the majority of reads norovi-rus-specific (median value 91%), The median read depth of the full genomes was 4679. As expected given the use of a capture array, there was not a strong correlation between these three measures of sequencing performance and the input Ct value with performance only dropping at the highest Ct values. Four samples showed lower total reads and lower norovirus-specific reads (Patient 4
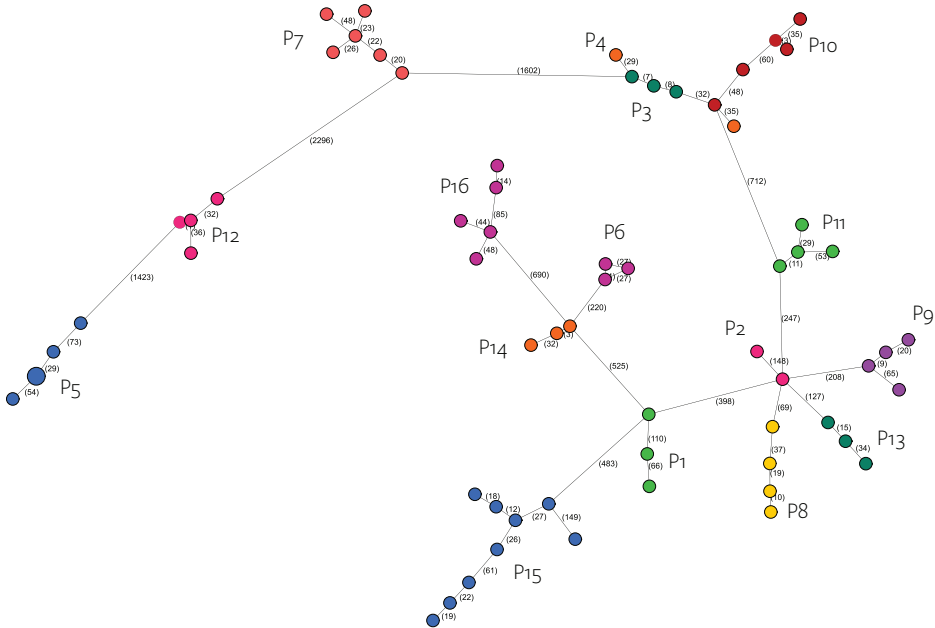
Figure 1 Minimum spanning haplotype network constructed from 65 norovirus genome sequences, showing the relationship between genomes within and between patients in the study. Each node represents a genome and nodes are coloured by patient of origin with the length of vertices represents number of nucleotide changes (indicated within brackets) between pairs of genomes. The size of the node is proportional to the number of genomes clustered in the node.

day 355, Patient 10 day 462, Patient 11 day 83, Patient 10 day 508). Several samples (Patient 4 day 355, Patient 9, day 121 and 176, Patient 10 day 508, Patient 12 (all four time points), and Patient 16 (day 0)) yielded more than one genome using *de novo* assembly suggesting either co-infection or technical contamination. Co-infection with another genotype from the study was excluded based on epidemiological data (sampling date) and clustering of samples in a minimum spanning tree, and technical contamination was the most likely explanation for these secondary genomes based on sequence batch number and genome coverage. In order to avoid over interpretation of the data, low coverage contigs were therefore removed from the analysis. As a measure of sample handling and quality control, a minimum spanning tree of all 65 genomes was constructed (Figure 1). Genomes clustered closely by patient in nearly all cases, although one group of patients (Patient 3, 4, 10) were infected with related viruses that showed some overlap in the tree. Maximum-likelihood phylogenetic trees of full genomes and each of the three ORFs were inferred (Supplementary Figure 2), showing similar consistent patterns of patient-derived genomes.

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Table 1 Cohort description

ID	Age	dT	Month/ year first sample	Complaints of gastro- enteritis since*	Group	Norovirus genotype	Underlying illness	Immunosup- pressive therapy
1	56	716	Jan 2009	Dec 2008	SOT	GII.P4 Apeldoorn 2007 - GII.4 Apeldoorn 2007	Kidney transplantation	MMF, Tacrolimus, corticosteroids
2	3	402	Mar 2012	Feb 2012	SOT	GII.P4 New Orleans 2009 - GII.4 New Orleans 2009	Kidney transplantation	MMF, Tracrolimus
3	72	122	Apr 2013	Apr 2013	SOT	GII.Pe - GII.4 Sydney 2012	Kidney transplantation	MMF, Tacrolimus, corticosteroids
4	44	355	Apr 2013	Mar 2013	SOT	GII.Pe - GII.4 Sydney 2012	Kidney trans- plantation	MMF, Tacrolimus, corticosteroids
5	66	441	May 2013	May 2013	SOT	GII.P7 - GII.6	Kidney trans- plantation	MMF, Tacrolimus, corticosteroids
6	55	204	Nov 2011	Nov 2011	SOT	GII.P4 Den Haag 2006b - GII.4 Den Haag 2006b	Kidney trans- plantation	MMF, Tacrolimus, corticosteroids
7	12	637	Feb 2011	Dec 2010	SOT	GII.P21 - GII.3	Kidney trans- plantation	MMF, Tacrolimus, corticosteroids
8	6	340	Jun 2012	Jun 2012	SOT	GII.P4 New Orleans 2009 - GII.4 New Orleans 2009	Kidney trans- plantation	MMF, Tacrolimus, corticosteroids
9	58	176	Aug 2013	Dec 2012	SOT	GII.P4 New Orleans 2009 - GII.4 Not assigned	Lung trans- plantation	MMF, Tacrolimus, corticosteroids
10	57	462	Jan 2013	Jul 2012	SOT	GII.Pe - GII.4 Sydney 2012	Lung trans- plantation	MMF, Tacrolimus, corticosteroids
11	35	413	Jan 2013	Jan 2013	SCT	GII.P4 New Orleans 2009 - GII.4 Sydney 2012	Allogeneic stem cell trans- plantation	MMF, corticos- teroids
12	36	76	Jul 2013	Jun 2013	He- mato	GII.P7 - GII.7	Follicular non-Hodgkin's lymphoma	Unknown
13	57	235	Dec 2010	Dec 2010	SCT	GII.P4 New Orleans 2009 - GII.4 New Orleans 2009	Allogeneic stem cell trans- plantation	Cyclosporine, corticosteroids
14	48	109	Jan 2008	Jan 2008	He- mato	GII.P4 Den Haag 2006b - GII.4 Den Haag 2006b	Chronic lymphocytic leukaemia	-
15	71	495	Jun 2009	May 2009	Other	GII.P4 Not assigned - GII.4 Hunter 2004	Good's syn- drome	-
16	62	451	Jun 2010	Aug 2004	Other	GII.P4 Not assigned - GII.4 Not assigned	Vasculitis	Cyclophospha- mide, Azathio- prine, corticos- teroids

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Abbreviations Table 1 ID = Patient identifier, dT = duration of chronic infection in days as defined by the time between first and last sample, SOT = Solid Organ Transplant , SCT = Stem Cell Transplant, Hemato = Hematologic disease, MMF = Mycophenolate mofetil

*as reported in the hospital patient database and subjected to the interpretation of the clinician

GII.4 variant
- US 95-96
- Farmington Hills 2002
- Hunter 2004
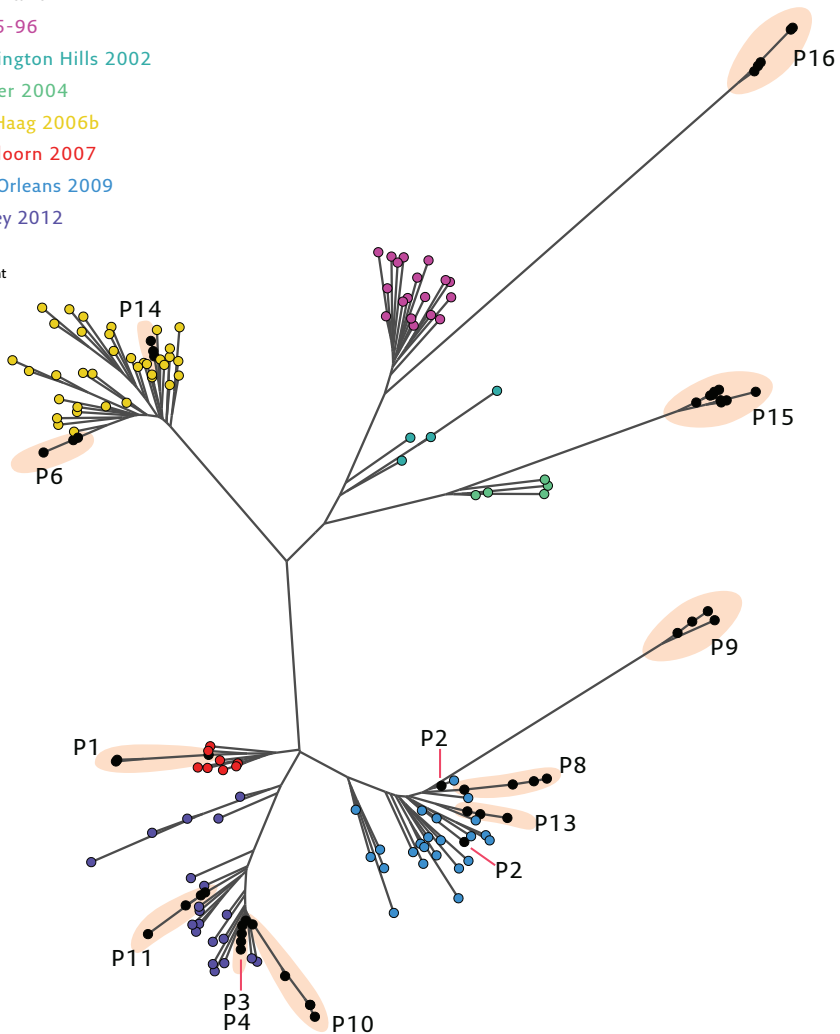- Den Haag 2006b
- Apeldoorn 2007
- New Orleans 2009
- Sydney 2012

0.01 nt

P16, P14, P6, P15, P9, P1, P2, P8, P13, P2, P11, P3, P4, P10

Figure 2 Maximum-likelihood phylogenetic tree constructed from 50 consensus VP1 nucleotide sequences of 13 patients with chronic norovirus GII.4 infection compared to 114 norovirus GII.4 VP1 GenBank reference sequences. The reference sequences were coloured by GII.4 variant, black nodes represent sequences from patients with chronic infection, and salmon coloured shading indicates sequences derived from identical patients.

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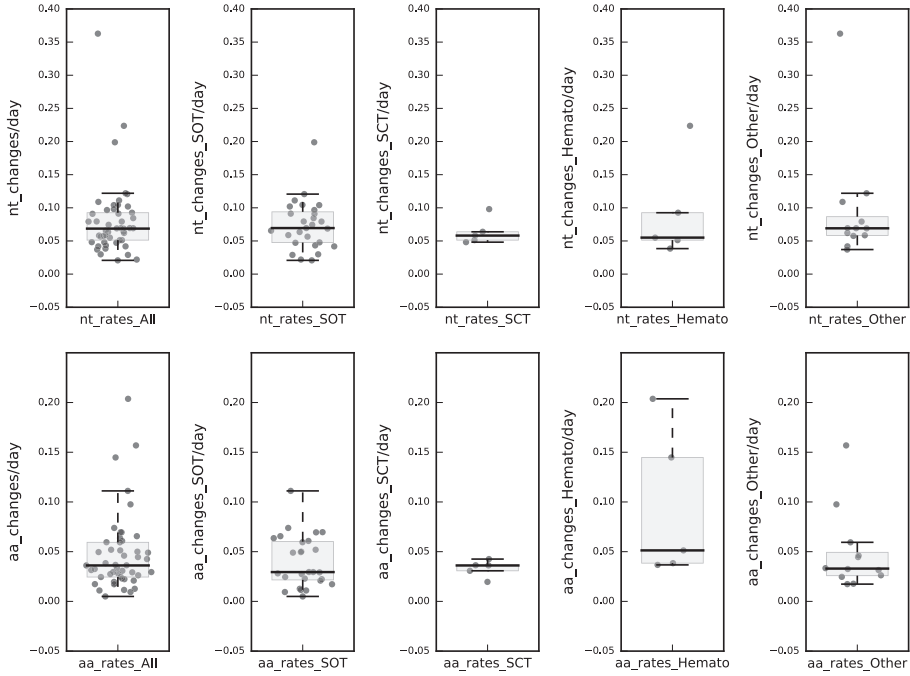
Figure 3 A) Within patient norovirus nucleotide and amino acid mutation rate. Average nucleotide and amino acid changes per day for norovirus full genome sequences derived from all 16 patients with chronic infection stratified per immune status (SOT = solid organ transplantation, SCT = stem cell transplantation, Hemato = hematologic disease). The changes were calculated by aligning nucleotide or protein sequences and counting the number of differences between the initial and the query sequence. The elements of the plots include the values from individual genomes (gray dots), the median value for the set (thick black line), the interquartile range for the set (grey rectangle) and the lower and upper quartiles (horizontal black lines above and below the rectangle).

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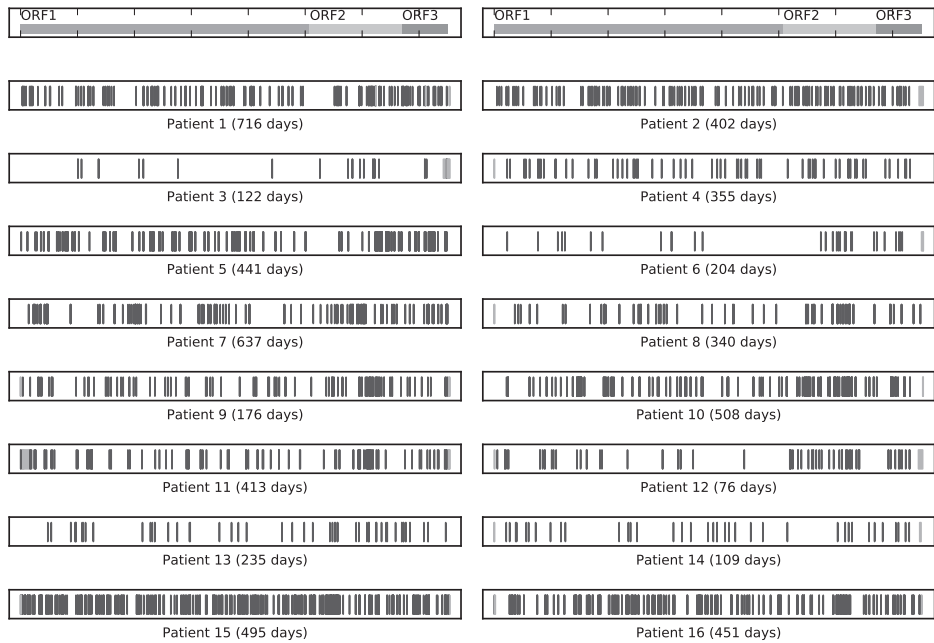
Figure 3 B) Accumulated nucleotide changes occurring in each patient plotted by genome position. For each patient, all nucleotide changes from the initial consensus genome sequence were plotted by position (dark gray bar, and gaps are shown in light grey). The total number of days of observation for each patient is listed after the patient id. A diagram showing positions of the three major norovirus open reading frames (ORF) is shown at the top of each column.

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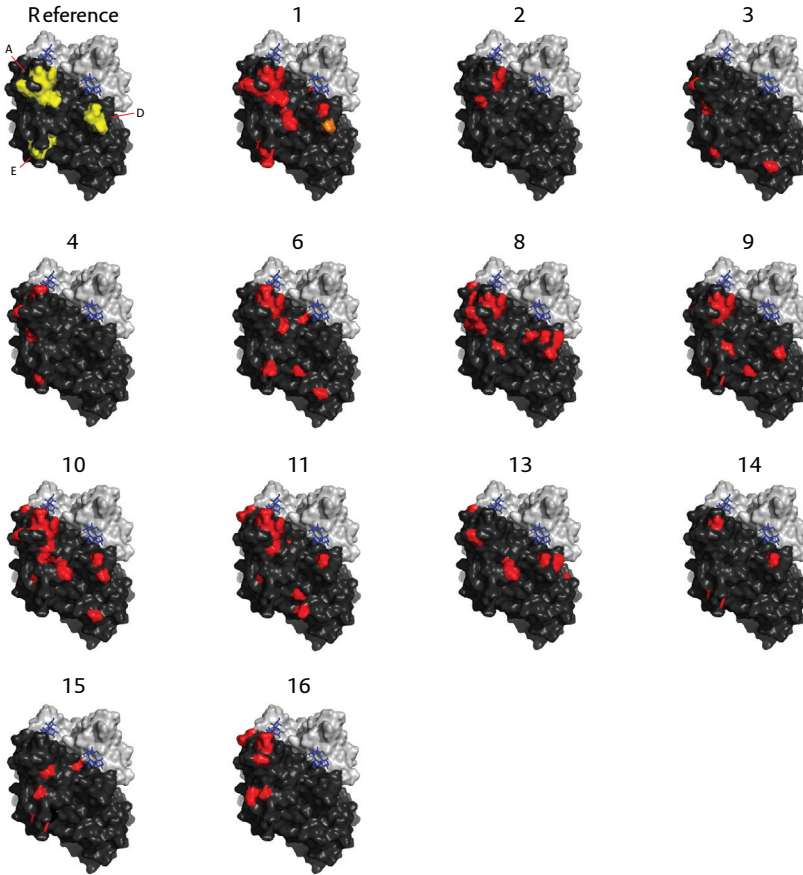
Figure 4 The norovirus P domain GII.4 Sydney 2012 dimer structure (PDB id 4OP7, download-ed from http://www.rcsb.org/pdb) with the within-host changes in aa residues that occurred between the first and last sample coloured in red and deletions in orange. The reference structure shows epitopes A, D, and E in yellow. Numbers above the protein models indicated study patient identifier numbers. Dark grey and grey indicate both monomers that form the P domain dimer and blue indicates a glycan structure that functions as the norovirus (co-) receptor. The PyMOL Molec-ular Graphics System version 1.8 (Schrödinger, LLC) was used to localise amino acid residues.

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PatientID	Sequence_ID	A 294	A 296	A 297	A 298	A 368	A 372	D 393	D 394	D 395	E 407	E 412	E 413
p1	E7800007_p1_d0	T	S	R	N	A	D	N	T	A	S	N	S
p1	E1300272_p1_d566	T	S	H	S	N	N	G	-	T	S	N	I
p1	E7800016_p1_d716	S	T	H	R	D	S	G	-	T	S	S	T
p2	E1300296_p2_d0	P	S	H	N	A	D	S	T	T	S	N	I
p2	E1300297_p2_d402	P	S	R	N	A	D	S	T	T	S	N	I
p3	E1300301_p3_d0	T	S	R	N	E	D	S	T	T	S	N	T
p3	E1300302_p3_d72	T	S	R	N	E	D	S	T	T	S	N	T
p3	E1300303_p3_d122	T	S	R	N	E	D	S	T	T	S	S	T
p4	E1300308_p4_d0	T	S	R	N	E	D	S	T	T	S	N	T
p4	E1300309_p4_d355	T	S	R	N	E	D	S	T	T	S	S	T
p6	E1300319_p6_d0	A	S	R	N	S	E	G	T	T	S	D	V
p6	E1300320_p6_d18	A	S	R	N	S	E	G	T	T	S	D	V
p6	E1300321_p6_d204	A	S	H	N	E	K	G	T	T	S	D	V
p8	E7800011_p8_d0	P	S	R	N	A	D	S	T	T	S	N	I
p8	E1300329_p8_d173	P	S	H	N	A	N	D	T	T	S	N	I
p8	E1300330_p8_d269	P	S	H	N	A	N	D	T	P	S	N	I
p8	E7800020_p8_d340	S	S	H	N	A	N	D	T	P	S	N	I
p9	E1300280_p9_d0	G	T	N	R	D	S	D	D	T	S	G	T
p9	E1300281_p9_d58	G	T	N	R	D	S	D	D	S	S	G	T
p9	E1300282_p9_d121	G	S	N	R	D	S	D	D	S	S	G	T
p9	E1300283_p9_d176	G	S	D	R	D	H	D	D	S	S	G	I
p10	E7800008_p10_d0	T	S	R	N	E	D	G	T	T	S	N	T
p10	E1300285_p10_d316	T	S	S	T	E	N	D	T	A	S	N	T
p10	E1300286_p10_d362	T	S	S	T	E	N	D	T	A	S	N	T
p10	E1300287_p10_d462	T	S	S	T	E	N	D	T	A	S	N	T
p10	E7800017_p10_d508	T	S	S	T	E	N	D	T	A	S	N	T
p11	E7800009_p11_d0	T	S	R	N	E	D	S	T	T	S	N	T
p11	E1300306_p11_d83	T	S	R	N	E	D	S	T	T	S	N	T
p11	E1300307_p11_d195	T	S	R	N	E	D	S	T	T	S	N	T
p11	E7800018_p11_d413	T	S	H	H	E	N	N	T	T	S	N	T
p13	E7800012_p13_d0	P	S	R	N	A	D	S	T	T	S	N	I
p13	E1300300_p13_d51	P	S	R	N	A	D	S	T	T	S	N	I
p13	E7800019_p13_d235	S	S	R	N	A	D	N	T	T	S	N	I
p14	E7800015_p14_d0	A	S	R	N	S	E	S	T	T	S	D	V
p14	E1300305_p14_d39	A	S	R	N	S	D	S	T	T	S	D	I
p14	E7800023_p14_d109	A	S	R	N	S	D	G	T	T	S	D	I
p15	E7800013_p15_d0	G	T	Q	S	S	S	S	T	T	E	D	S
p15	E1300263_p15_d41	G	T	Q	S	S	S	S	T	T	E	D	S
p15	E1300264_p15_d81	G	T	Q	T	S	S	S	T	T	E	D	S
p15	E1300265_p15_d102	G	T	Q	T	S	S	S	T	T	D	D	S
p15	E1300266_p15_d174	G	T	Q	T	S	N	S	T	T	E	D	S
p15	E1300267_p15_d215	G	T	Q	T	S	S	S	T	T	D	D	S
p15	E1300268_p15_d419	G	T	Q	T	S	S	S	T	T	D	D	N
p15	E1300269_p15_d476	G	T	Q	T	S	S	G	T	T	N	D	N
p15	E7800021_p15_d495	G	T	Q	T	S	S	S	T	T	E	D	N
p16	E7800014_p16_d0	G	T	H	D	H	N	D	-	N	D	G	T
p16	E1300288_p16_d101	G	T	H	D	H	T	D	-	N	D	G	T
p16	E1300289_p16_d202	G	T	H	D	H	N	D	-	N	D	G	T
p16	E1300290_p16_d403	G	T	H	D	H	T	D	-	N	D	G	T
p16	E7800022_p16_d451	G	T	H	D	H	T	D	-	N	D	G	T

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Figure 5 Within-host aa changes of VP1 blockade epitope A, D, and E among longitudinal samples of 13 patients with chronic GII.4 infection[28]. Amino acid changes of GII.4 variant reference sequences are indicated on top. Gray shading indicates changed acid residues for each column and patient.
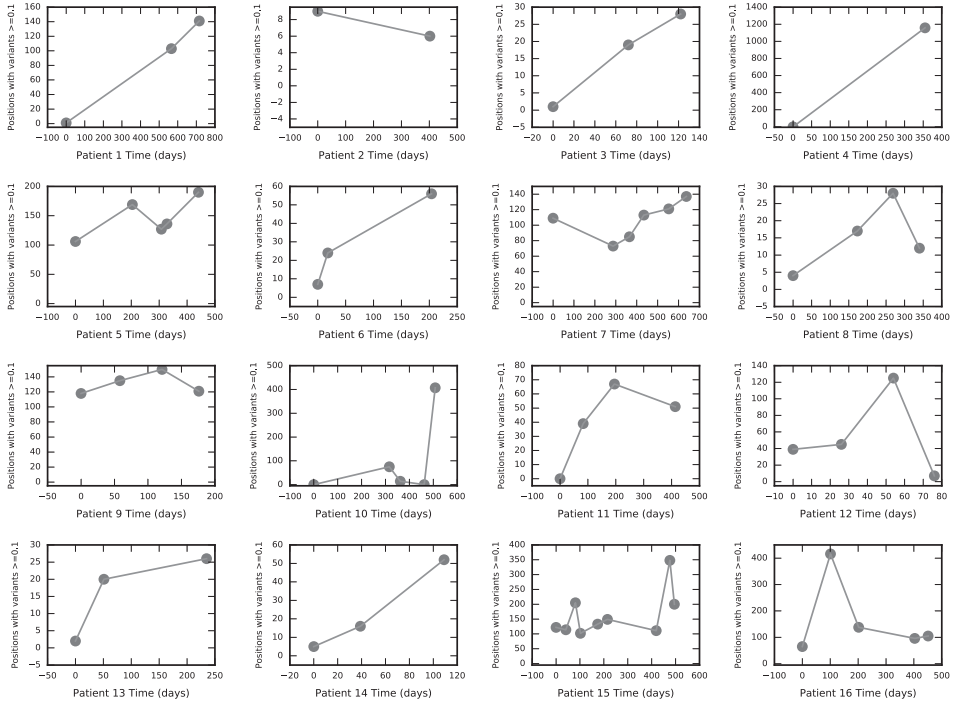
Figure 6 Minor variant content. For each sample, the specific minor variant content was determined by mapping all quality-controlled reads to the final consensus genome from that sample (See Methods section). Only sites with >100 fold coverage and reads with Phred quality score of >35 were reported. Positions with non-consensus nucleotide at >10% frequency were graphed and the total number of positions with variants in each category are listed at the left of each panel.

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Within-host genetic evolution compared to viral genetic diversity in the general population

To study within-host virus evolution, ORF2 consensus sequences from chronically infected patients were compared to ORF2 sequences derived from GenBank reference sequences using a maximum parsimony analysis (GII.4: Figure 2, and GII.3, GII.6, GII.7: Supplementary Figure 3A, 3B, and 3C, respectively). Ten of 13 GII.4 infected patients (76.9%) were infected with viruses similar to known GII.4 variant clusters (Figure 2). However, three of 13 (23.1%) first patient specimens contained a virus with large genetic distance (97 of 1623 nt (6,0%), 82 of 1623 nt (5,1%), and 135 of 1623 nt (8,3%), patients 9, 15, and 16, respectively) to closest known circulating GII.4 strain (as determined by nt BLAST search). Remarkably, these patients were first sampled in August 2013, June 2009, and June 2010 (Patients 9, 15, and 16, respectively), but were infected with viruses closest to GII.4 New Orleans 2009, GII.4 Hunter, and GII.4 US95/96, respectively, which were predominant years before these patients were sampled. Patients 5, 7, and 12 infected with non-GII.4 norovirus strains were infected with strains with large genetic distance to reference strains, but this can be explained -in part- due to a limited number of available reference sequences (Supplementary Figures 3A, 3B and 3C).

Viral mutation rate and the effect of immune status

Full genome consensus sequences were used to determine the within-patient average nt and aa changes per day (Figure 3A). The nt changes per day among individual patients showed variation from 0.03 to 0.37 nt / day, and 0.01 to 0.21 aa / day and although variation among individuals was high, no significant mutation rate differences were observed between immune status groups (solid organ transplantation, stem cell transplantation, hematologic disease, or other). The positions of nucleotide changes across the norovirus genomes were examined. All nucleotide changes occurring during the entire observation period were plotted for each patient (Figure 3B). Changes were observed throughout the genome.

Mutation hotspots in the P domain of VP1

A structure of the norovirus GII.4 Sydney 2012 P domain has been generated previously[27] and this model was used to locate within-host aa changes for each GII.4 infected patient individually (Figure 4). The majority of the observed aa changes were on the predicted outer surface of the P domain complex at or near epitope A, D, and E. A few aa changes were located more deeply within the P-domain structure: patient 3 V385A, patient 8 V385I, patient 9 P305L, patient 11 R286K, and patient 13 L452I. To further study the effect

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of within-host evolution on antigenic evolution and receptor specificity, aa positions for blockade epitopes A, D, and E, known for GII.4[28], are shown for 13 GII.4 patients over time (Figure 5). The exact location of antigenic epitopes for genotype GII.3, GII.6, and GII.7 are unknown and therefore changes in the epitopes of the virus populations of patients (5, 7, and 12) were not determined. All patients showed to contain a virus population with at least one aa change during follow up in the antigenic epitopes (Figure 5). The insertion of epitope D position 394 is found in a highly variable loop region, located at the top of the P2 domain, and present among all GII.4 variants since Farmington Hills 2002[29, 30]. Patient 16 was infected with a strain most related to GII.4 US95_96 and therefore lacks this insertion in all specimens. Remarkably, patient 1 was infected with GII.4 Apeldoorn 2007, initially contained the insertion at position 394, but lost it by day 566. Consensus ORF2 sequences of virus populations were also inspected for within-host mutations of the receptor binding pocket site I, II, and III: (GII.4: aa 343-347, aa 374, and aa 442-443, GII.6: aa 360 – 363, aa 389, and aa 452, GII3: aa 357-360, aa 386, and aa 450 – 451, GII.7: aa 349 – 351, aa 378, and aa 446 – 447, respectively)[31]. None of the 16 patients contained a virus population that showed within-host aa changes in the receptor binding pocket sites and therefore none of the virus populations changed receptor specificity (data not shown).

Viral non-consensus minor variants
The minor variant diversity was plotted over time to study whether the minor variant diversity can be used as an indicator for chronic infection (Figure 6). Although the viral diversity is variable over time, the majority of the 16 patients showed increases in minor variant content over the observation period: 6 of 16 patients showed consistent increase in the minor variant content with each longitudinal sample having more variants than the previous sample and 13 of 16 patients had a final time point with more variants than first time point. Six patients started with high variant content (40 or more sites with >10% minor variant content, patients 5, 7, 9, 12, 15, 16) and may indicate that these patients were infected for some time before the initial sampling. Variation was observed throughout the genome suggesting that the variation was a consequence of a random process and not due to accumulation of changes as a consequence of selective pressure on specific proteins (data not shown). Furthermore, the minor variant content should be interpreted with caution as several samples showed evidence of contamination (see paragraph quality control).

Discussion

The aim of this study was to describe norovirus within-host evolution and diversity in chronically infected and immunosuppressed patients. We show that immunocompromised patients were initially infected by viruses which can be phylogenetically linked to strains found in the general population. However, over the observation time, the within-host virus populations evolve into virus populations that are genetically distinct and with mutations across the genome including the antigenic domains. Immunocompromised patients therefore contain a reservoir of viruses that are genetically and possibly antigenically distinct from viruses circulating in the general population. While similar observations have been reported for individual or small patient groups, we show that all chronic norovirus patients that we identified in our hospital had evidence of evolving viruses[12, 13, 15, 32-34].

Three of the GII.4 infected immunocompromised patients (patient: 9, 15, and 16) shed viruses with a large genetic distance to any norovirus available in public databases. These patients were likely infected some time (possibly years) before the initial sampling of this study or alternatively were infected by a strain transmitted from another immunocompromised patient (which was also not represented in the public database). For patient 16 there is evidence of long-term unexplained symptoms of gastroenteritis before the initial sample in June 2010. This patient was reported to have symptoms of gastroenteritis for the six previous years and in agreement with this observation the sample from 2010 contained a virus strain with nearest ancestor to GII.4 US95/96 which was predominantly detected in the surveillance until 2002[5]. Thus, potentially this patient was infected with the same strain for at least six years before the sampling in the current study. The VP1 sequence from the last available samples of patient 9, 15, and 16 showed a similarity of 94%, 95%, 92% respectively with the closest known strains. There is not a strict distance criterion for new GII.4 variants, but currently recognised successive variants have nt similarity of >95%[33, 35]. However, the novel variants found in this study do not justify a new name since they are not yet found as epidemic strain in two geographic distinct regions[35].

It remains to be determined whether new virus variants found in immuno-compromised patients are of risk for other patients or the general population. Transmission between immunocompromised patients has been reported earlier, but only in an early stage of infection with a maximum of 17 days after initial diagnosis[36, 37]. We have tried to retrospectively find transmission events in this study by linking epidemiological information (sampling date and hospital ward)

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of positive samples from immunocompromised patients to positive samples obtained from other patients, but have not yet found transmission events (data not shown). This can however be a result of under-sampling of the hospital patient population and staff, as previously described[38]. It could also be that the viruses found in immunocompromised patients are highly adapted to their host and/or have a lower fitness due to the limited immune pressure and are therefore not easily transmitted to other humans. This could theoretically mean that immunocompromised patients are only infectious in an early stage of infection when the virus still maintains features necessary for infection in immunocompetent hosts.

A specific capture method was used to deep sequence the whole norovirus genome from clinical samples without the need for additional amplification steps; these methods have been successfully implemented for other viruses and bacteria[17, 39-41]. The method provided a high coverage across the full norovirus genome allowing for minor variant analysis with high sensitivity, in addition to analysis of the full genomes.

The majority of patients had a viral population with an increasing minor variant content over time and therefore we concluded that an increased minor variant content is an indication for chronic infection as suggested by others[12, 42]. Patients shedding viruses with large genetic distance (patients 9, 15, and 16) also had a highly diverse variant content on day 0, which supports our conclusion that these patients were already infected some time before the initial sampling. Other patients already showed high diversity in the earliest samples or a more variable pattern over time, which might be explained by a missed norovirus infection with the earliest sequenced sample actually obtained after an unknown period of virus evolution in the suppressed patient, disease progression, or changes in the immunosuppressive therapy. The technical contamination as detected in some of the specimens, and quite commonly observed with Illumina deep sequencing, is a limitation of the quasi species analysis as presented in this study, but we have corrected for this as described in the methods section.

There is currently no registered antiviral therapy available for norovirus infection and patients with dehydration or malnutrition caused by norovirus infection can only be treated with supportive treatment. It has been observed that a temporary pause in the immunosuppressive therapy could allow transient immune system recovery and help to clear the virus, but this should be done with great care since this entails a risk for organ rejection and is not an option for patients who are immunocompromised due to other causes[43]. In other studies, a limited

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number of infected immunocompromised patients received experimental enterally administered immunoglobulin therapy as a treatment against chronic norovirus infection with mixed success rates[44-46]. With the results of this study we hypothesize that the success of immunoglobulin treatment depends on the antigenic distance between the within-host strain and the virus population circulating at the moment of immunoglobulin harvesting. Given the high within-host mutation rates as shown in this study, immunoglobulin treatment may only be successful when it is produced from plasma batches from local patients recovering from similar viruses and administered in an early stage of infection. Future prospective studies are needed to address this issue.

The number of immunocompromised patients has largely increased in the last few decades due to an increase in the number of allogeneic transplants. It was shown here that novel norovirus variants are developed in the immunocompromised host and future prospective studies are needed to assess the risks of transmission of this new strains to other immunocompromised patients and the general population and subsequent consequences for hospital infection control guidelines. In view of the frequent reports of hospital acquired norovirus infection, and the potential consequences in patients at risk for chronic norovirus infection, low-threshold screening for infection should be considered in high risk patient wards.

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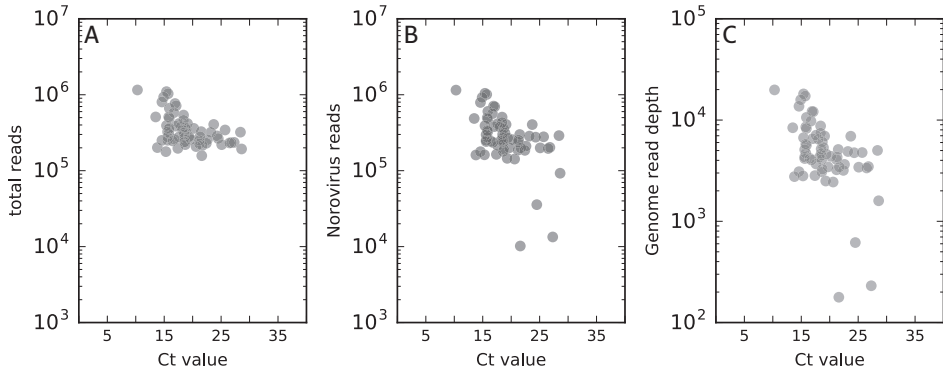
Supplementary Figure 1 Read yield quality control. The total number of reads for the 65 samples (A), the number of norovirus specific reads (B), and the average read depth of the resulting sample genome (C), were plotted as a function of the RT-PCR cycle threshold value Ct.
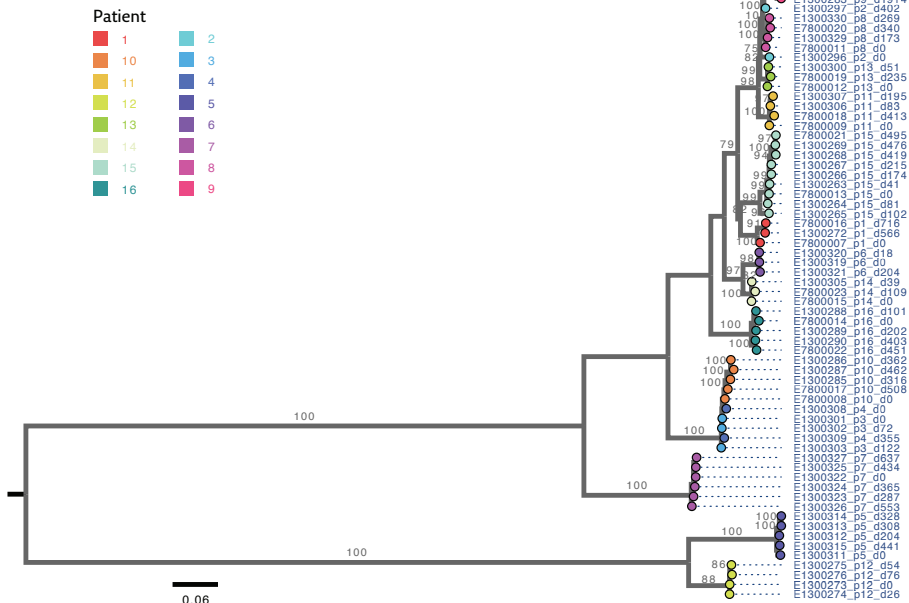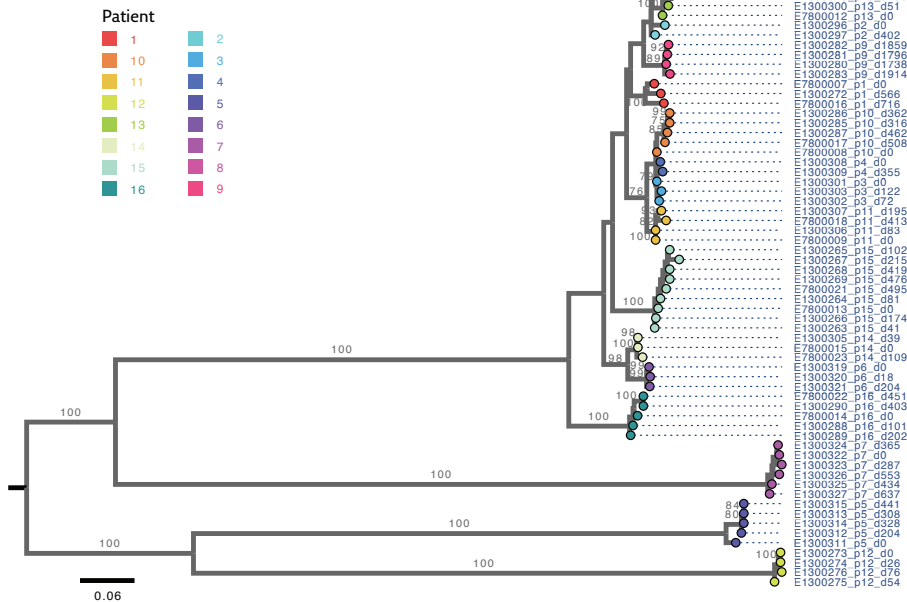
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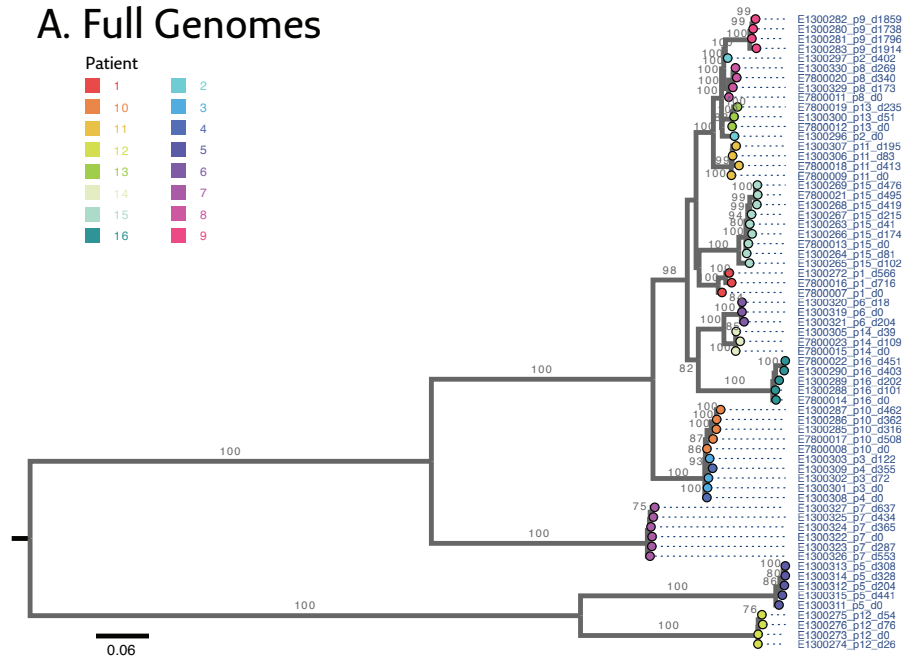
A. Full Genomes

C. ORF2
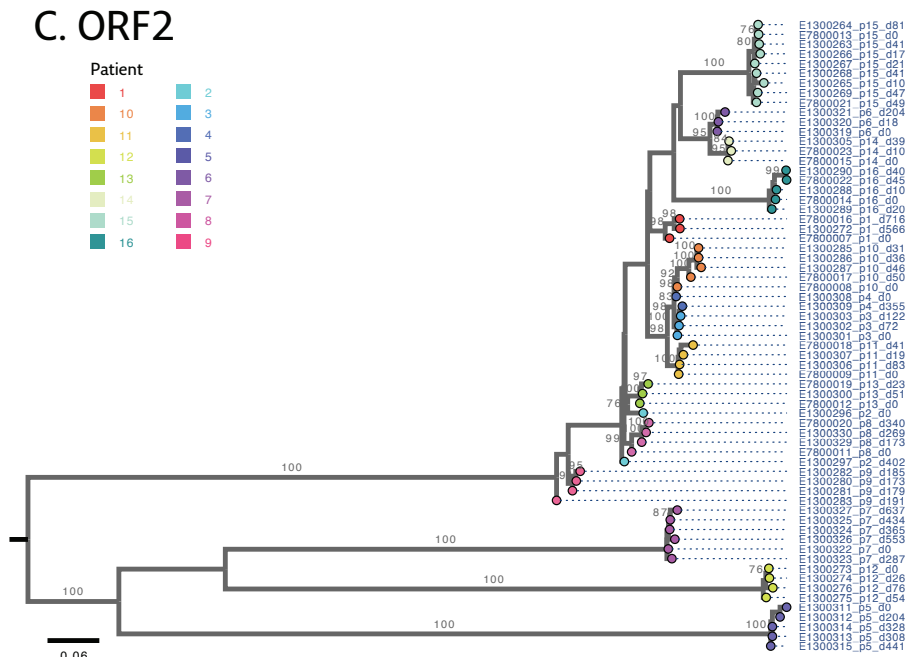
Supplementary Figure 2 Maximum-likelihood phylogenetic trees constructed from 65 consensus full genome and ORF1, ORF2, and ORF3 nucleotide sequences obtained from 16 patients with chronic norovirus infection.

B. ORF1

D. ORF3
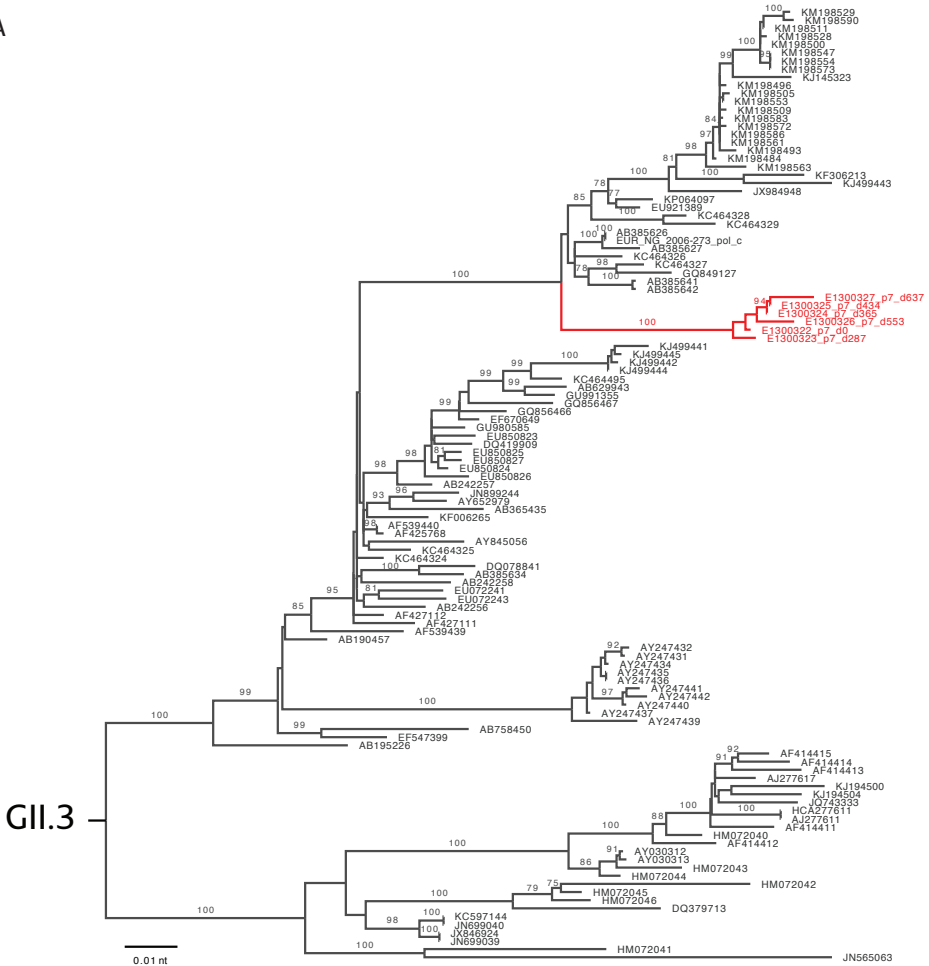
Supplementary Figure 3 Maximum-likelihood phylogenetic tree constructed from 15 consensus VP1 nucleotide sequences of three patients (indicated in red) infected by norovirus GII.3 (patient 7 - day 0, 287, 365, 434, 533, 637) (**A**), GII.6 (patient 5 – day 0, 204, 308, 328, 441) (**B**), or GII.7 (patient 12 – day 0, 26, 54, 76) (**C**), compared to VP1 GenBank reference sequences (indicated in black).

B

GII.6

0.04 nt

C

GII.7

0.01 nt

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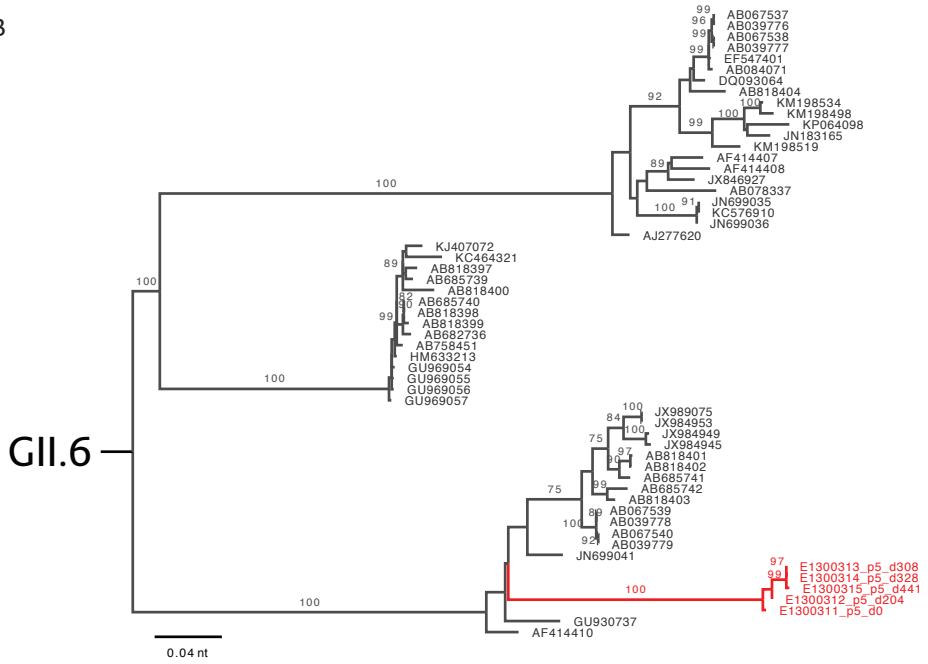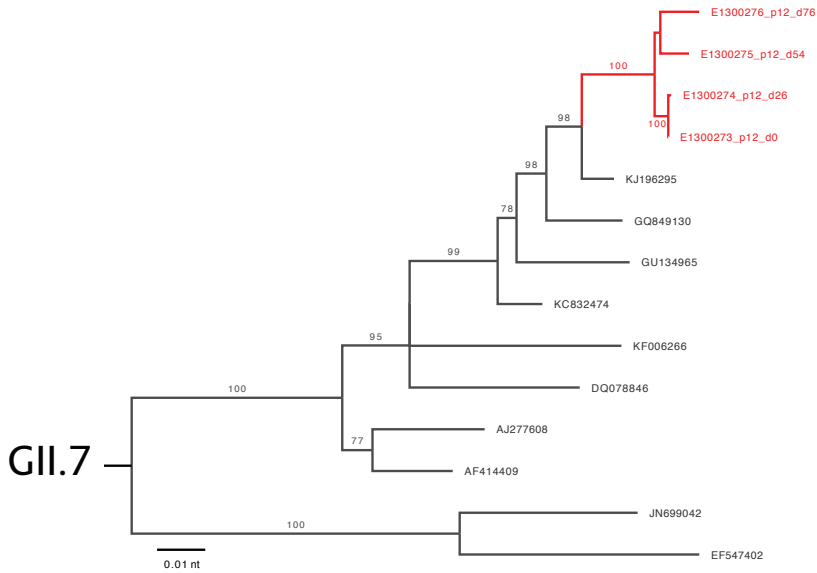
Summarizing discussion

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The general aim of this thesis was to get a better understanding of the global norovirus diversity, with a focus on the role of chronic norovirus infection on virus diversity, antigenic variation and evolution. The obtained knowledge can be used to predict severe norovirus outbreak seasons, is useful for improvement of hospital hygiene and infection control guidelines, and is relevant for future vaccine development.

Large norovirus genetic diversity

The norovirus genetic diversity is extremely large as outlined in the introduction of this thesis. New norovirus diversity is discovered on a regular basis: novel genogroups, genotypes, recombinants within and between genotypes, and antigenic drift variants have been found and described recently[1-7]. The distribution and incidence of norovirus genotypes changes over time and by geographic region, with GII.4 as the predominant genotype responsible for 70-80% of the outbreaks detected worldwide during the last two decades[8-12]. Changes in the norovirus epidemiology have a large effect on the society since the emergence of novel drift variants goes hand in hand with an increase in the number of reported outbreaks, including the number of hospitalizations and deaths related to acute gastroenteritis[13-15]. We show in **Chapter 2.1** that an increase in global norovirus reports late 2012 was related to the emergence of the novel GII.4 Sydney 2012 variant. This new GII.4 Sydney 2012 variant was the 6th in a row of variants with a global distribution since the start of norovirus molecular surveillance in the mid-1990s: US95/96, Farmington Hills 2002, Hunter 2004, Den Haag 2006b, New Orleans 2009, and followed by GII.4 Sydney 2012[5, 16]. GII.4 Sydney 2012 remained the predominant strain during the following years, but this pattern was disrupted in some countries in Asia late 2014. A novel GII.P17-GII.17 (GII.17 Kawasaki 2014) replaced GII.4 among outbreaks of acute gastroenteritis in China and Japan during the Northern hemisphere winter of 2014/2015 and was detected widely in European countries in after years, as described in **Chapter 2.2** and **Chapter 3** and by others[17-19]. This is a unique observation since the replacement of the predominant GII.4 has never been observed by molecular surveillance. It is an important finding since norovirus research always had a focus on GII.4 during the last two decades, although genotype replacements may have occurred in history before the molecular surveillance started (mid 1990s) as shown in **Chapter 4** by using a multiplex serological assay. Interestingly, GII.17 has not yet become the predominant genotype worldwide in the summer of 2017 and no new GII.4 variants have originated since the emergence of GII.4 Sydney 2012 in the 2012/2013 winter season, in contrast to what was expected following the observed pattern of the last twenty years. Some known genotypes

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have caused (local) epidemics recently, like GII.P2-GII.2 and GII.P16-GII.2, and a new recombinant GII.4 Sydney 2012 (GII.16-GII.4 Sydney 2012)[20-23]. The replacement of the polymerase gene for the latter appears to have led to more efficient replication and/or transmissibility[6, 24, 25]. These observed rapid changes in norovirus genetic diversity raise concerns about the current vaccine candidates under development with GII.4 components and raise questions regarding the origin of norovirus diversity and the underlying mechanisms and evolutionary driving forces.

Origin of norovirus diversity

The norovirus GII.4 evolutionary driving forces are well described: the human population builds up immunity against the outer surface of the major capsid protein which drives the selection of virus variants that can evade infection blocking antibodies via antigenic drift[26]. Population immunity is therefore the main norovirus evolutionary driving force. This process occurs in a stepwise manner, frequently referred to as epochal evolution[9]. The number of VP1 amino acid (aa) changes between successive global GII.4 variants lies in the range of 21-25 aa[9]. In addition, GII.4 drift variants accumulate multiple mutations in the minor capsid protein compared to their precursors, likely to maintain capsid stability, and these mutations add up to the already large genetic jump of the VP1[27]. The main question here is: how and in which host does the virus acquire the mutations needed for a new drift variant? It is most likely that new GII.4 variants originate in the human population since GII.4 is widespread in the human population, evolutionary driving forces are driven by population immunity, and natural GII.4 infections among animals are considered to be very rare[28]. Despite its high mutation rate, it is not likely that the viruses could develop all mutations required for a new variant over the course of a normal infection in a single immunocompetent host since norovirus shedding usually lasts only four weeks. If we assume that multiple hosts are required for the development of new drift variants, it is unexpected that intermediate GII.4 variants are scarcely detected in the surveillance, although this could potentially be explained by the absence of norovirus surveillance in most countries. Norovirus surveillance is absent in almost all of Africa, South America, and in Asian countries except China and Japan as shown in Chapter 3, and only a very small fraction of sporadic cases and outbreaks is investigated by sequencing methods. Precursor drift variants could therefore be potentially missed by surveillance, especially when these strains are only spread in a restricted geographical area. An alternative hypothesis is that novel norovirus variants are developed within individuals with a chronic infection due to a diminished immune system. These chronic shedders can shed virus for

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multiple years in extremely high quantities, which would provide enough time to accumulate all required mutations to develop a new drift variant within a single host. The potential for chronic norovirus shedding was considered to be rare until recently. In **Chapter 5** we showed that long term virus shedding is more common among solid organ transplant patients than originally thought and we more than doubled the number of patients described in the literature. To get a better understanding of the potential for virus drift within prolonged shedders we studied the within host evolution of chronic shedders using next generation sequencing technology in **Chapter 6**. We showed that viruses within these patients evolve to highly divergent strains, with large genetic distance to known virus clusters including antigenic changes at the blockade epitopes over time. We therefore concluded that immunocompromised patients contain a reservoir for new norovirus genetic diversity. It however needs to be determined whether these new strains are transmissible to other individuals and are able to cause large epidemics or pandemics.

It has been suggested that the level of immunosuppression of the host may determine the extent of viral evolution[29]. While the viral populations of severely immunocompromised individuals show a very high viral diversity in contrast to viral populations found among healthy individuals, they might not be very well adapted to escape host immunity from healthy individuals. Infected individuals with an intermediate level of immunity (i.e. elderly, malnourished) may have the optimal intermediate level of immune suppression to generate a complex quasispecies and drive virus evolution. A recent study tried to investigate the hypothesis whether immunocompromised individuals could be a source of new GII.4 variants and concluded that rapidly evolving norovirus populations among immunocompromised host do not significantly contribute to the overall norovirus evolution[30]. This conclusion is based on a mathematical SIRS model that estimates the contribution of the immunocompromised and immunocompetent subpopulations to the overall pathogen evolution. A sensitivity analysis (testing different scenarios) was performed to determine the robustness of the model, especially for parameters with large uncertainty like the norovirus basic reproduction number, duration of immunity, and proportion of immunocompromised patients. The authors finally conclude that: "…,despite an increased rate of nucleotide substitution and an extended duration of infection, the rarity of immunocompromised hosts in the population limits their influence to broader NoV dynamics, which are still dominated by the lower evolutionary rates apparent in the general population"[30]. Or in other words: the immunocompromised population is too small compared to the immunocompetent population, to have an effect on the overall norovirus evolution – even with very

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high mutation rates and long lasting infections as found in chronic shedders. There are some problems with this conclusion. Viral population dynamics are extremely complex and each individual mutation can have an effect on the viral life cycle, receptor specificity, transmission efficiency, viral fitness, or stability of virus particles, but the mathematical model used by the authors did count each mutation equal, which is an extreme simplification of the processes underlying virus population dynamics. Furthermore, it is not the accumulated genetic diversity among all immunocompromised individuals in the population that forms a new GII.4 variant as suggested by the model of the authors, but a very rare event that only occurs in an individual patient every 2-3 years somewhere in the world. Thus the authors used unsuitable methods to study this hypothesis in my opinion, and future studies using sequence confirmation are needed to determine the transmissibility of new variants found in immunocompromised individuals as suggested earlier.

Recombination due to template switching of the RNA dependent viral polymerase (RdVP) is another important driving force of norovirus evolution and occurs most frequently at the ORF1/ORF2 breakpoint[31]. Novel recombinants, like the GII.P16 - GII.4 Sydney 2012, originate from hosts infected by multiple norovirus strains. Foodborne outbreaks are often caused by a mixture of strains and are therefore a potential source of novel recombinant strains[32]. Long lasting infections, as recently observed in immunocompromised patients, may also constitute a risk for recombination events since these patients could easily obtain a secondary infection.

Implications for vaccine development

Two vaccine candidates are currently in phase I and II clinical trials: an intramuscular bivalent vaccine based on GI.1 and GII.4 virus like particles (VLP) (Takeda vaccines, Tokyo, Japan) and a monovalent oral vaccine based on GI.1 virus protein 1 (VP1) (Vaxart, San Francisco, USA)[33]. The efficacy of the monovalent vaccine has not been tested yet and two doses of the intramuscular bivalent vaccine was shown to reduce the risk for getting gastroenteritis with 32% towards a homologous strain in a randomized, double-blind, placebo-controlled study[34]. The bivalent vaccine has a protective effect to a homologous strain under ideal circumstances, but it is less effective than the vaccine effectiveness of the currently licensed influenza vaccine, and shows that norovirus vaccine development is complex and that significant improvements need to be made before the vaccine can be accepted on the market. It further remains to be determined how long the vaccine provides protection, how efficacious it is in individuals with a diminished immune system (e.g. elderly

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people), and whether it can provide protection against heterologous strains. Heterologous protection is of major importance since multiple norovirus genotypes are circulating within the human population simultaneously as shown in chapter 3 and since it is likely not feasible to include all known human genotypes in the vaccine due to related high costs. We only have limited understanding of the human immune response and the level of protection upon natural norovirus infection. Some studies suggest that natural infection with a genotype protects with limited duration against (symptomatic) infection with other genotypes of the same genogroup (within genogroup heterologous protection), while other studies could not repeat these results, potentially due to a limited studied population size[35-37]. A birth cohort study from Peru found that repeated infections with a genotype of the same genogroup were common within a 2-year time span, but repeated infections with the same genotype were rare[38]. Patterns of norovirus immune protection are complex and may resemble patterns of antigenic relatedness rather than genetic similarity at the genogroup level. Prospective studies need to address whether natural infection protects from subsequent infection with heterologous genotypes, and whether current vaccine candidates can simulate natural infection. Norovirus vaccine design should take into account that major changes in the norovirus epidemiology require major and time constrained updates of the antigenic composition of the vaccine, especially if heterologous immune protection is not feasible with current vaccine technology. A recent study showed that IgA antibodies better block norovirus binding to histo-blood group antigens than their IgG isotype-switched variants[39]. Thus mucosal IgA may play a key role in immunity to norovirus and new adjuvants and novel administration techniques could potentially improve mucosal immunity to norovirus and therefore enhance the efficacy of the vaccine[40, 41]. Norovirus vaccine research has always been hampered by the lack of a cell culturing system, which prohibited the development of neutralization assays to study immune correlates of protection. HBGA blocking assays using virus like particles (VLPs) are used as a surrogate, and have been useful to get a better understanding of factors influencing binding of norovirus to the host cell HBGAs, but may not fully correlate with protection from disease[42]. The discovery that human noroviruses can be cultured in B cells is a recent breakthrough in the norovirus research field and an important finding on the road to an effective norovirus vaccine on the market[43, 44].

Future perspective on chronic shedders

Norovirus infection is common among immunocompromised individuals and can become a chronic infection as shown in **chapter 5 and 6**. Patients with chronic norovirus infection shed virus in extremely high quantities for multiple

years. An important and not well studied question is whether chronic norovirus infection causes a symptomatic or asymptomatic infection. Symptoms of gastroenteritis are known to be common among the immunocompromised patient populations and can have multiple causes, including immunosuppressive treatment, host-versus-graft disease, viral, bacterial or parasitic infections, or a combination of these. Norovirus infection has always been considered to be an acute disease and of short-term duration, and therefore not related to persistent symptoms of gastroenteritis. The symptoms of the patients enrolled in our studies were not systematically recorded since both studies were retrospective, and we could only retrieve symptoms from the hospital medical patient record database. However, some of the patients enrolled in our studies were positive for norovirus and reported to have long-term and/or severe complaints of gastroenteritis corresponding to the period of norovirus shedding, suggesting that norovirus was the causative agent. Two other studies recently reported severe diarrheal disease among immunocompromised patients related to norovirus infection, which indicates that this is an under recognized syndrome which potentially highly affects the quality of life of already severely ill patients[45, 46]. Future studies need to determine the incidence of long-term gastroenteritis among the norovirus infected immunocompromised patient population. Higher awareness among clinicians and frequent sampling could distinguish norovirus related diarrhoea from other causes of diarrhoea, and help to guide treatment. Treatment options include reduction or modification of the immunosuppressive therapy, experimental immunoglobulin treatment), and possibly off label nitazoxanide treatment[47-50]. Novel antiviral compounds are furthermore under development[51-53].

An important question for hospital hygiene and infection control guidelines is whether noroviruses found in immunocompromised patients are transmissible to other patients or the general population. Immunocompromised patients with norovirus infection are not isolated from other patients in the current hospital practice, as suggested earlier for all viral pathogens causing gastroenteritis[54]. Chronic shedders shed virus in extremely large quantities and, given the low infectious dose required to induce infection, it is not unlikely that these patients transmit the virus to e.g. other immunocompromised patients in the hospital. Evidence on the transmission of norovirus between immunocompromised patients is scarce since this question has not been systematically studied. We therefore advocate to enhance surveillance in this vulnerable patient population.

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Concluding remarks

In conclusion, the norovirus genetic diversity is continuously evolving and the global dominance of GII.4 for at least two decades is under pressure. The observed changes in the norovirus molecular epidemiology with frequent emergence of GII.4 drift variants, emergence of novel recombinants, and predominant genotype switches require a norovirus vaccine that can be easily updated within a limited time span. The drift variants observed among immunocompromised individuals may constitute a risk for other immunocompromised individuals and might be a reservoir of new norovirus variation for the general population. Future studies need to address the risk of transmission between immunocompromised individuals and between immunocompromised individuals and healthy individuals. Immunocompromised individuals with chronic norovirus infection may suffer from persistent symptoms of diarrhoea with a consequent serious loss of quality of life, and future studies need to address the severity and incidence of chronic diarrhoea and how these patients can be treated to eliminate infection.

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Summary

Noroviruses belong to the family of *Caliciviridae* and cause acute gastroenteritis. The genetic diversity within the genus *Norovirus* is extremely large and novel genotypes, recombinants within and between genotypes, and antigenic drift variants are regularly discovered. The distribution and incidence of norovirus genotypes changes over time and by geographic region, with GII.4 as the predominant genotype responsible for approximately 70% of the outbreaks detected worldwide. This thesis contributes to a better understanding of the global norovirus diversity, and the role of chronic norovirus infection among immunocompromised individuals on virus diversity, antigenic variation and evolution. We describe the emergence of a novel GII.4 drift variant and novel GII.17 in chapter 2.1 and 2.2, respectively. Chapter 3 provides an overview of the global norovirus molecular epidemiology using data obtained by the NoroNet surveillance network in the last ten years. Chapter 4 shows that GII.4 was not predominant before the wide spread use of norovirus molecular typing methods in the mid-1990s. Chapter 5 shows that chronic norovirus infection is common among solid organ transplant patients and more than doubled the number of described patients in literature. Finally, chapter 6 shows that virus populations in the immunocompromised host are genetically distinct from viruses circulating in the general population. These hosts therefore may contain a reservoir of newly emerging strains and future studies need to address whether these new strains can be transmitted to other immunocompromised patients or individuals in the general population. Norovirus vaccines are currently tested in clinical trials. The observed global changes in the norovirus molecular epidemiology require a norovirus vaccine with broad immune protection and an antigenic component that can be easily updated within a limited time span. Immunocompromised patients with chronic norovirus infection may suffer of persistent symptoms of diarrhoea with a consequent severe loss of quality of life and future studies need to address how these patients can be treated to eliminate infection.

Nederlandse samenvatting

Norovirussen behoren tot de familie *Caliciviridae* en veroorzaken acute gastro-enteritis. De genetische diversiteit van het genus *Norovirus* is extreem groot en nieuwe genotypes, recombinanten binnen en tussen genotypes, en drift varianten worden regelmatig ontdekt. De geografische verspreiding en incidentie van norovirus genotypes veranderen in de tijd, waarbij genogroup II genotype 4 (GII.4) het meest voorkomende genotype is en verantwoordelijk voor ongeveer 70% van de uitbraken wereldwijd. Deze thesis draagt bij aan een beter begrip van de wereldwijde genetische diversiteit van norovirussen, en de rol van chronische infectie bij patiënten met een immuundeficiëntie op de virusdiversiteit, de antigene variatie en de evolutie. We beschrijven de uitbraak van een nieuwe GII.4 drift- variant en een nieuwe GII.17 in respectievelijk hoofdstuk 2.1 en 2.2. Hoofdstuk 3 geeft een overzicht van de wereldwijde moleculaire epidemiologie van norovirussen met data verzameld via het NoroNet netwerk in de afgelopen tien jaar. Hoofdstuk 4 laat zien dat GII.4 niet het meest voorkomende genotype was voor het wijdverspreide gebruik van moleculaire typeringsmethodes in het midden van de jaren negentig. Hoofdstuk 5 laat zien dat een chronische norovirus infectie algemeen is bij orgaantransplantatie-patiënten en deze studie verdubbelde het aantal in de literatuur beschreven patiënten ruim. Ten slotte laat hoofdstuk 6 zien dat virus populaties bij patiënten met een immuundeficiëntie genetisch afwijken van de virussen die worden gevonden in de algemene populatie. Deze patiënten zijn daardoor mogelijk een reservoir van nieuwe uitbraakstammen. Toekomstige studies moeten uitwijzen of deze virusstammen kunnen worden verspreid naar andere patiënten of individuen in de algemene bevolking. Norovirus vaccins worden momenteel getest in klinische studies. De geobserveerde veranderingen in de moleculaire epidemiologie vragen om een norovirus vaccin met brede immuun-bescherming en een makkelijk en snel aan te passen antigene component. Patiënten met een immuundeficiëntie en een chronische norovirus infectie hebben mogelijk last van chronische diarree met ernstige gevolgen voor de kwaliteit van leven en toekomstige studies moeten uitwijzen hoe deze patiënten het beste behandeld kunnen worden om van de infectie te genezen.

PhD Portfolio

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Education

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2012 – 2016   PhD training, National Institute for Public Health and the Environment (RIVM), Bilthoven / Erasmus MC, Rotterdam, the Netherlands.

Courses

2012   Virology course, Erasmus MC, Rotterdam
2012   Analysis of microarray gene expression data using R/BioC and web tools, Erasmus MC, Rotterdam
2013   Advanced immunology course, Utrecht University
2013   Analysis of serological data, RIVM, Bilthoven
2014   BLS-3 course on laboratory biosafety and biosecurity

Scientific conferences and meetings

2012   Norovirus and other Caliciviruses on the rise, Lubeck, Germany
2013   VIRGO meeting, Scheveningen (oral presentation)
2013   Caliciviruses congress, Beijng, China (oral presentation)
2015   VIRGO meeting, Scheveningen (oral presentation)

Teaching

2013   Supervision of university master student
2014   Viruskenner coach high school students

Other activities

2013 - 2016 Reviewer Pathogens, Emerging Infectious Diseases, Journal of
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2015   Voluntary deployment Dutch Mobile Ebola Laboratory, Sinje, Liberia

Publications

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Curriculum Vitae

Janko van Beek was born in Alkemade, the Netherlands, on November 23th, 1983. He graduated from high school in 2001 (Bonaventura college, Leiden), started to study BSc Biology in 2003, and graduated MSc cell biology at the Wageningen University in 2009. He developed special interest in the field of infectious diseases and public health during his study and selected and worked on related topics for his Bachelor thesis, Master thesis, and Master internship: "The natural reservoir of SARS-coronavirus and the mechanism of viral interspecies transmission', 'The role of the PB1-F2 protein on virus-host interactions of influenza A virus', and 'The aetiology of community acquired pneumonia in the Netherlands', respectively. Janko got his first job at the National Institute for Public Health and the Environment (RIVM) and assisted with the laboratory response to the emergence of the new influenza A H1N1 in 2009. He continued to work at the RIVM and Erasmus Medical Center as a PhD student on this thesis in 2012, with prof dr. Marion Koopmans as promoter and dr. Harry Vennema as co-promoter. Janko was selected for the European Public Health Microbiology Training (EUPHEM) of the European Centre for Disease Prevention and Control (ECDC) and started his fellowship at the National Institute for Health and Welfare (THL) institute in Helsinki, Finland, in September 2016.

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