Human norovirus emergence and circulation in humans and animals

Nele Cora Villabruna

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Human norovirus emergence and circulation in humans and animals

De verspreiding van humaan norovirus in mens en dier

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Nele Cora Villabruna
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Erasmus University Rotterdam

Doctoral Committee

Promotor:	Prof. dr. M.P.G. Koopmans
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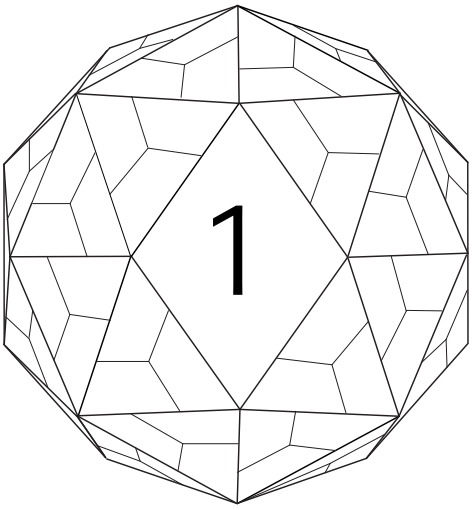


General introduction

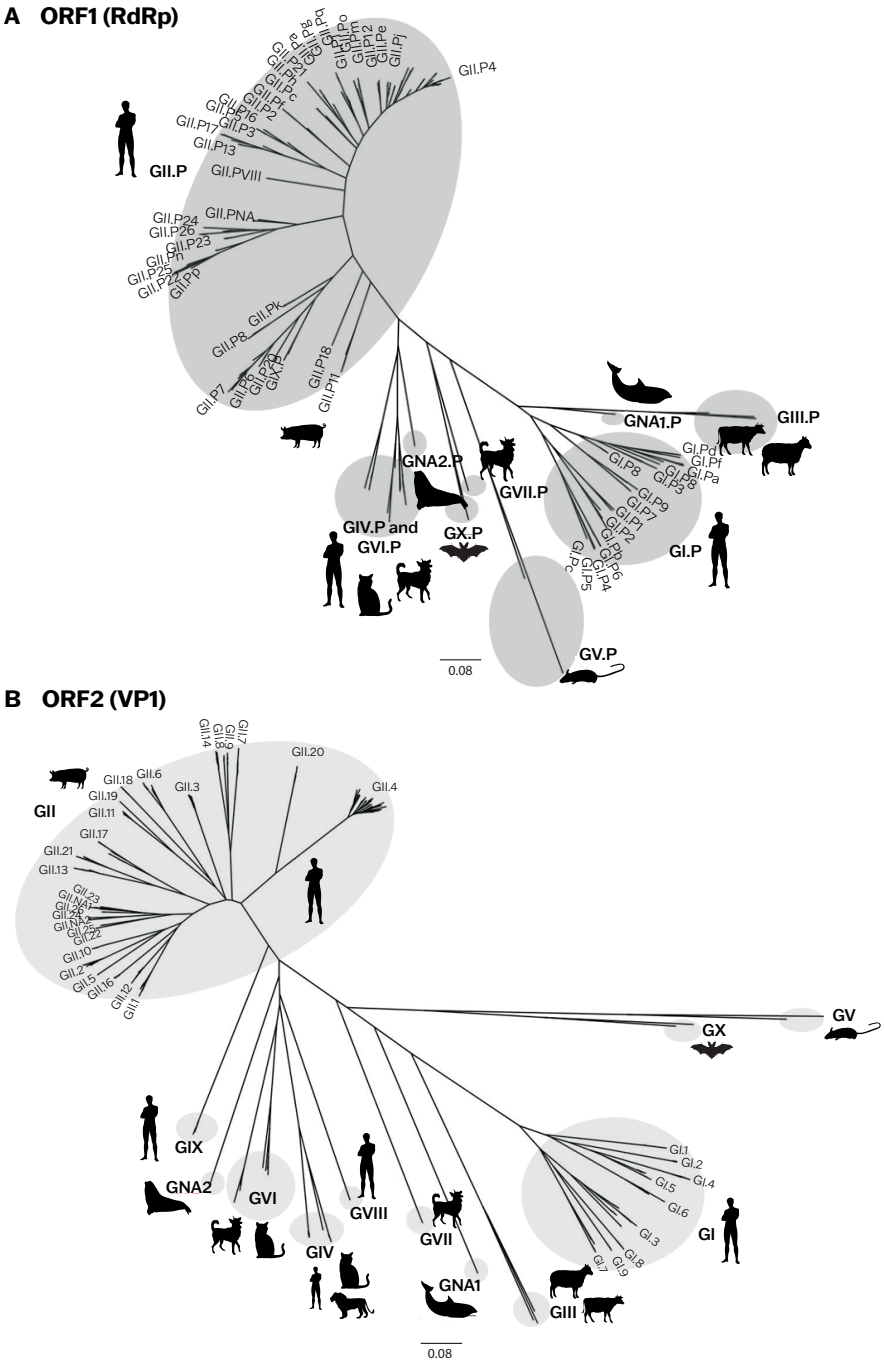
Norovirus background

Norovirus infections are the leading cause of acute gastroenteritis globally. The burden of norovirus-related illness is a significant public health concern, causing an estimated 648 million infections and approximately 200,000 deaths per year [1]. This burden of disease is especially high in young children in developing nations.

Norovirus is one of 11 genera of the *Caliciviridae* family, infecting humans and non-human animals. Mammals are infected by the genera *Lagovirus*, *Nebovirus*, *Recovirus*, *Sapovirus*, *Valovirus* and *Vesivirus*, birds by *Bavovirus* and *Nacovirus*, and fish by *Minovirus* and *Salovirus* [2]. Based on sequence similarity, noroviruses are grouped into 10 genogroups (GI-GX) and further divided into more than 48 genotypes, of which some include several variants and lineages (**Fig. 1**) [3]. The genomic nucleotide similarity between viruses from different genogroups is 51%–56% and within the same genotype 69%-97% [4, 5]. Viruses within genogroups GI, GII, GIV (GIV.1), GVIII, and GIX infect humans, while viruses of other genogroups have been found in a broad range of animal hosts, such as pigs (GII.11, GII.18, and GII.19), cattle (GIII.1 and GIII.2), sheep (GIII.3), rodents (GV), cats (GIV.2 and GVI), a lion (GIV.2), dogs (GVI and GVII), harbor porpoises (GNA1), sea lions (GNA2), and bats (GX).

The norovirus virion (infectious virus particle) is 24-40 nm in diameter and encapsidates a 7.5 kb positive-sense single-stranded RNA genome with a poly (A) tail (**Fig. 2**) [6]. The genome is typically organized in three open reading frames (ORF1-ORF3) [7]. ORF1 encodes for a polyprotein that is enzymatically cleaved by the viral protease into six proteins. Starting from the N-terminal, these are p48 (NS1/2), a nucleoside triphosphatase (NTPase, NS3), p22 (NS4), VPg (NS5), protease (NS6), and the RNA-dependent RNA polymerase (RdRp, NS7). P48, NTPase, and p22 co-localized in various cell cultures with the viral replication machinery, endocytic pathway components, and cellular organelles [8-10]. VPg is, similar to its homolog in picornaviruses, a protein primer that is important for initiation of RNA synthesis by the RdRp and the translation initiation complex [11, 12]. The two N-terminal proteins p48 and p22 are more diverse between caliciviruses and share as little as 60% amino acid similarity within on genogroup and ~40% between genogroups. The NTPase, VPg, protease, and the RdRp, in contrast, are more conserved, sharing >80% amino acid similarity. The RdRp crystal structure has been solved for GI.1, GII.4, GV, Rabbit haemorrhagic disease virus (*Lagovirus*), and Sapovirus [13-17]. These RdRps share

Figure 1. Norovirus phylogeny of ORF1 and ORF2. Due to frequent recombination between ORF1 and ORF2, a dual typing system is used to identify the polymerase and the capsid type. (**A**) Phylogenetic tree based on partial RdRp sequences of norovirus genogroups (P-groups) and genotypes (P-types). P-groups and P-types with only a single sequence are referred to as non-assigned (NA). (**B**) Phylogenetic tree based on VP1, which are classified into ten genogroups (GI-GX) based on their capsid amino acid sequence. Two additional genogroups are not assigned yet (NA). The figure is adapted from Chhabra ***et al.*** [3].


A ORF1 (RdRp)
GII.P4
GII.P
GII.P1
GII.P3
GII.P13
GII.PVIII
GII.PNA
GII.P24
GII.P26
GII.P23
GII.P21
GII.P25
GII.P22
GII.P9
GII.Pk
GII.P8
GII.P7
GII.P20
GII.P11
GII.P18
GNA1.P
GIII.P
GNA2.P
GVII.P
GIV.P and GVI.P
GX.P
GI.P
GV.P
0.08
B ORF2 (VP1)
GII
GII.20
GII.4
GII.18
GII.6
GII.19
GII.3
GII.11
GII.17
GII.21
GII.13
GII.10
GII.2
GII.5
GII.16
GII.12
GII.1
GV
GX
GIX
GNA2
GVI
GVIII
GVII
GIV
GNA1
GIII
GI
GI.1
GI.2
GI.4
GI.5
GI.6
GI.3
GI.8
GI.9
GI.7
0.08

36%-60% sequence identity and a high degree of structural similarity. The structure resembles a right hand with fingers, palm, and thumb domains, of which the palm domain is the most conserved and includes the active sites DYTxxD and YGDD [18]. Human norovirus RdRps crystalizes in dimers, indicating that this might be the active form. The error-prone nature of RdRps and the resulting low fidelity is a key driver for norovirus evolution.

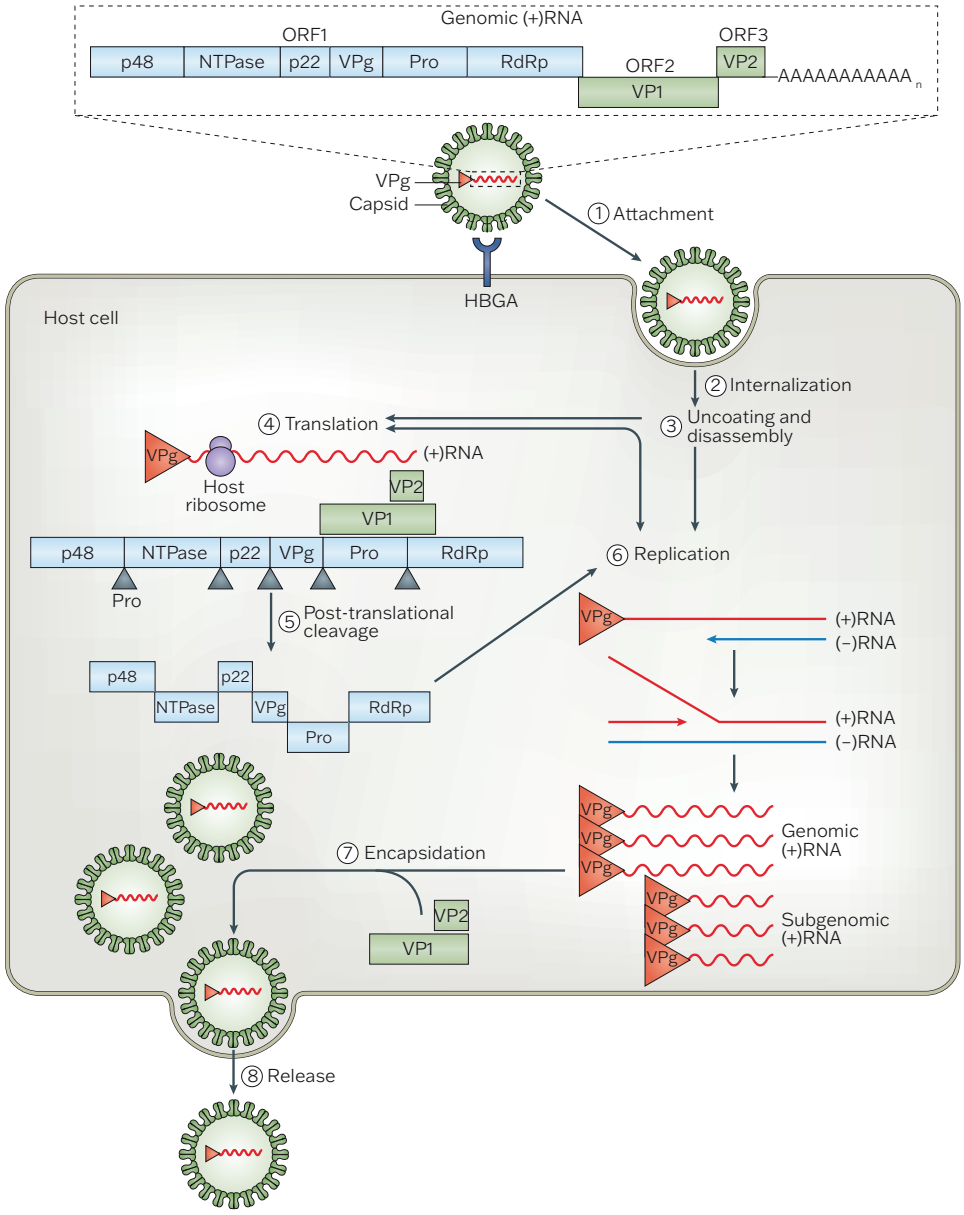
ORF2 and ORF3 are translated from genomic and subgenomic RNA and encode the major and minor viral capsid proteins (VP1 and VP2). VP1 forms dimers of which 90 dimers assemble into the outer capsid [19]. VP1 is composed of two domains, the conserved shell (S) domain, and the protruding (P) domain, which is further divided into the P1 and P2 subdomains [20]. The P2 domain contains the receptor-binding site and the antigenic epitopes A-H [21, 22]. In contrast, only a few copies of VP2 are found on the interior of each virion, increasing its stability and possibly acting as a link between capsid and viral RNA [23-25]. In addition, a recent study has found that, upon receptor binding, twelve copies of the feline calicivirus VP2 form a portal-like assembly leading to a pore in the capsid [26]. It is hypothesized that the viral RNA is released through this pore into the host cell cytosol.

Transmission and epidemiology

Noroviruses are transmitted via the fecal-oral or oral-oral route, either through direct contact with infected individuals or indirectly through exposure to contaminated food, water, or surfaces [27-29]. In addition, transmission via vomitus of infected individuals and subsequent ingestion of virus particles has also been reported [30].

National and international surveillance networks (e.g., NoroNet and CaliciNet) have provided insights into differences between norovirus genotypes and genogroups regarding modes of transmission and outbreak settings. This indicates that the genotypical differences are related to phenotypical differences. For example, 76% of GII.4 outbreaks are reported from residential institutions and hospitals [31]. While 43% of non-GII.4 and GI outbreaks occur in these settings, the rest is reported from outbreaks occurring in hotels, restaurants, and schools. Differences are also detected with regards to transmission routes; the GII.4 genotype is predominantly transmitted person-to-person, while other genotypes (GI.3, GI.6, GI.7, GII.3, GII.6, and GII.12),

Figure 2. Genome organization and replication cycle of human noroviruses. The norovirus genome has three open reading frames (ORFs) that encode a polyprotein and the major and minor capsid proteins VP1 and VP2, respectively. The positive-sense single-stranded RNA (+ssRNA) genome is encapsulated in a capsid consisting of VP1 and a few copies of VP2. The virus attaches to the host cell surface via interactions between VP1 and the host's histo-blood group antigens (HBGAs) (**step 1**) and is subsequently internalized by the cell, uncoated, and disassembled (**steps 2**, **3**), by mechanisms not well understood yet. Once the +ssRNA genome is released into the cytoplasm, it is transcribed and translated.



The host translation factors are recruited by the virus protein VPg, which covalently binds to the 5' end of the genome and mediates translation (**step 4**). The ORF1 polyprotein is co- and post-translationally cleaved by the virus protease into stable precursor intermediates, subsequently resulting in six proteins (**step 5**). The viral genome is replicated via the synthesis of an intermediate −ssRNA, which is used as template for new genomic and subgenomic +ssRNA **(step 6**). Subgenomic +ssRNA encodes only ORF2 and ORF3 and leads to increased production of VP1 and VP2. During encapsidation genomic +ssRNA is packaged into new virions (**step 7**), which are released and subsequently initiate a new round of infection (**step 8**). Figure from de Graaf *et al.* [6].

are more often associated with foodborne outbreaks [32]. In addition, GI genotypes are more frequently detected in waterborne outbreaks than GII genotypes [29, 31, 33]. Differences are also seen between patient age groups. GII.4 causes the majority of outbreaks and sporadic cases in both adults and children, but several non-GII.4 genotypes (e.g., GII.3, GII.6, GII.7, GII.8, and GII.12), are regularly found in young children [34-40]. In contrast, norovirus diversity in sporadic AGE cases in adolescents and adults is less well studied [34, 41, 42]. Children younger than 5 years also infect a higher number of people in community outbreaks [43, 44]. This could be explained by higher contact rates or lower levels of hygiene.

In conclusion, several studies have shown that noroviruses vary with regards to transmission route and outbreak setting. Furthermore, some genotypes are found more commonly in children (i.e., GII.3) than in adults. Which host or virus factors contribute to these genotype and genogroup differences is not well understood.

Disease and tropism

Norovirus infection starts by ingestion of infectious virus particles and their passing through the intestinal tract. After an incubation time of 1-2 days, symptoms present that include diarrhea, vomiting, nausea, abdominal cramps, and fever, which on average last 1-6 days [45, 46]. The exact mechanism of these various symptoms and the underlying norovirus infection of the intestine is not fully understood. It is further not clear if tissue and cell tropism, as well as symptom duration and severity, differ between genotypes. Symptom onset is associated with histopathological changes of the small intestine that includes villous blunting, infiltration of the lamina propria by immune cells, loss of nuclear polarity, and flattening of the intestinal epithelium [47, 48]. Diarrhea is thought to be driven by temporary leakage caused by reduction of tight junctions, decrease in epithelial resistance, and increased epithelial apoptosis [49]. During rotavirus infection, a secreted viral enterotoxin, NSP4, is important for the onset of diarrhea, and inoculation with NSP4 alone is enough to induce diarrhea [50]. Norovirus illness has been speculated to also involve an enterotoxin but this has not been confirmed yet.

In animals, the link between norovirus infection and disease is less well understood. Similar to humans, animal noroviruses have been detected in symptomatic (diarrheic) and asymptomatic animals. Upon inoculation with human noroviruses, animal models including piglets, non-human primates, mice, and calves develop diarrhea albeit not in every study (reviewed in [51]).

While noroviruses are known to infect the small intestine, the precise cell tropism is still being investigated. Depending on host and genogroup or genotype, two different patterns of cell tropism are described in the literature: The first one being cells in the epithelia of the small intestine and the second one being immune cells in the lamina

propria of the small intestine or secondary lymphoid organs (**Fig. 3**). Information on cell tropism in humans is restricted to immunocompromised patients and stems from immunohistochemistry of biopsies from acute and chronic norovirus infections [47, 52]. In these intestinal tissues, viral components (RNA, structural proteins, and non-structural proteins) were observed in epithelial enterocytes and enteroendocrine cells of villi in the duodenum, jejunum, and ileum. Additionally, these viral components were detected in macrophages, dendritic cells, and T cells in the lamina propria. A similar tropism was detected in animals that had been experimentally inoculated with human noroviruses: intestinal enterocytes and macrophage-like cells in the lamina propria in gnotobiotic pigs and calves [53-55], macrophages, lymphocytes, and dendritic cells in the stomach, intestine, spleen, tonsils, and lymph nodes of miniature piglets [56], dendritic cells in the intestine of chimpanzees [57] and macrophage-like cells in the intestine, liver, and spleen of immunocompromised mice [58].

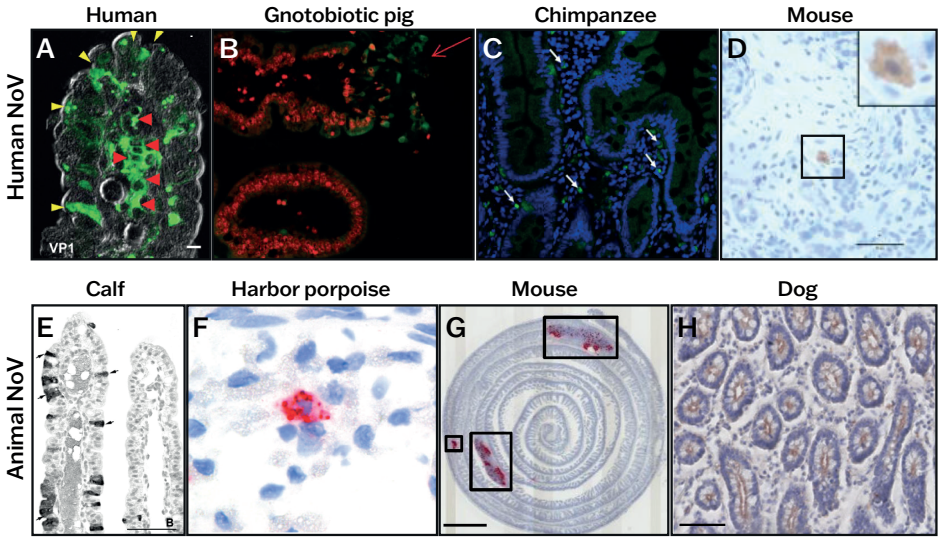


Figure 3. Tropism of human and animal noroviruses in different hosts. (**A**) Norovirus VP1 (**green**) in epithelial (**yellow arrows**) and lamina propria cells (**red arrows**) in the jejunum of an immunocompromised patient with chronic norovirus infection. Scale bar, 10 µm [47]. (**B**) GII.4 VP1 staining in epithelial cells of the villi and exfoliated enterocytes (**arrow**) in the duodenum of a gnotobiotic pig 3 dpi [54]. (**C**) GI.1 antigen in DC-SIGN positive dendritic cells (**arrows**) in the lamina propria of duodenum of a chimpanzee 4 dpi. Magnification 400× [57]. (**D**) Human norovirus capsid protein staining in macrophage-like cells in the small intestine of a mouse 24 hpi (intraperitoneal). Scale bar, 50 µm [58]. (**E**) GIII.1 antigen in epithelial cells (**arrows**) of mid jejunum of a calf 12 hpi. Scale bar, 100 µm [61]. (**F**) Staining of GNA1 RNA in enterocytes of naturally infected harbor porpoise. Magnification 100× [62]. (**G**) Staining of GV RNA in the gut-associated lymphoid tissue of small intestine (Swiss role) of infected mouse 1 dpi. Scale bar, 1 mm [59]. (**H**) Binding of canine GVI VLPs to epithelial cells of dog duodenum tissue. Scale bar, 50 µm [170].

For animal noroviruses, the tropism has only been investigated for GV, GIII, and GNA1 (**Fig. 3**). Depending on the strain, murine noroviruses infect either tuft cells in the intestine or macrophages, dendritic cells, B cells and T cells in the gut-associated lymphoid tissue [59, 60]. For GIII and GNA1 intestinal epithelial cells are the target [61, 62]. The identified tropism in biopsies is supported by the finding that human and murine noroviruses can be cultured in human intestinal organoids and murine macrophages, respectively [63, 64]. Furthermore, human noroviruses can be cultured in B cells, although *in vivo* host replication has not been shown [65].

In conclusion, the small intestine is the main site of norovirus replication in humans and animals. But questions remain regarding the exact cellular tropism and whether differences in tropism lead to differences in symptoms or disease outcomes.

Host susceptibility

Noroviruses are highly transmissible due to their resistance on surfaces as well as their low infectious dose [66]. But despite the high transmissibility, a subgroup of individuals does not become infected or symptomatic after exposure to certain norovirus strains [67-69]. This partial resistance has been associated with histo-blood group antigens (HBGAs), the sugars that determine the blood type. These terminal sugars of carbohydrate chains are linked to glycoproteins or glycolipids on red blood cells and various tissues, including the epithelial cells of the gastrointestinal tract [70, 71], and are secreted by these cells into the mucosa and saliva [72]. Their synthesis is mediated by α1,2-fucosyltransferases (FUT) and glycosyltransferases that are under the control of *FUT2* (secretor), *FUT3* (Lewis), and *ABO(H)* genes. The sequential addition of sugar groups to precursor structures results in the H, A, B, and Lewis antigens, which, when expressed on erythrocytes, define the blood type (O, A, B) [72]. Depending on geographical location, between 5%-20% of the human population, does not express a functional FUT2 enzyme, thereby lacking the H-antigen-based structures on their intestinal epithelium and in their saliva. These non-secretors are resistant to norovirus infection with certain strains [67-69, 73, 74] but not others [75, 76]. The important role that HBGAs play in norovirus susceptibility has been confirmed by volunteer studies, epidemiological data (reviewed in [77]), and also in the recently developed human intestinal organoid system [78]. Knock-out of the *FUT2* gene in secretor organoids diminished viral infection whereas knock-in of a functional *FUT2* into non-secretor organoids rendered them susceptible [78]. In animal experiments, the association between HBGA phenotype and susceptibility has only been studied for pigs [79]. Pigs expressing the A and H antigen on their intestinal tissue (A+/H+) were more susceptible to norovirus infection than pigs that did not express these antigens (A-/H-). A+/H+ pigs were more likely to develop symptoms, to seroconvert, and to shed virus compared to A-/H- pigs.

In conclusion, for some genotypes (e.g., GII.4), the HBGAs have a well-documented effect on host susceptibility. For other genotypes, the host HBGA phenotype and secretor status does not impact norovirus susceptibility, indicating that other, yet unknown, host factors are involved.

Host immune response

Why some norovirus infections lead to severe illness while others result in mild or no symptoms, is not fully understood since the correlates of protection are still under investigation and might vary between genotypes. Knowledge on the immune response against noroviruses stems predominantly from challenge studies and clinical trials, and the duration of protective immunity is still debated. In early studies, volunteers that were symptomatically infected with noroviruses could be reinfected after 27 months [80]. Based on a mathematical model assuming a population immunity level of 30%-45%, immunity has been predicted to last 4-9 years [43, 67, 81].

The sites that elicit an anti-norovirus antibody response, the antigenic epitopes, are predominantly located in the P2 domain of VP1 but the presence of epitopes on other viral proteins, cannot be excluded. The best understood protective mechanism of antibodies is their ability to block the interaction between the virus and its receptor, thereby inhibiting virus entry into the host cells and subsequently preventing infection. The development of the organoid culturing system has allowed virus neutralization in cell culture [82]. But more commonly, sera blocking of the HBGA-norovirus interaction is used as an approximation. HBGA blocking antibodies in sera have been found to correlate with protection from illness and infection [83-85]. Norovirus-specific B memory cells, as well as IgA from serum, feces, or saliva, were also recorded to protect from disease development, severity, or duration of virus shedding [67, 86-90]. While blocking antibodies have been detected against several genotypes, the actual HBGA-binding site as well as the antigenic epitopes A-H have only been mapped for GII.4 variants (**Fig. 4**) [21, 22, 91].

The IgG seroprevalence in adults is between 80%-100% with most studies investigating GI.1, GII.3, or GII.4. Most people will experience multiple norovirus infections during their lifetime and the cross-reactivity between genotypes from the same genogroup makes it challenging to determine the norovirus infection history on a genotype-specific level [75]. Questions remain regarding the extent of cross-reactivity between the many genotypes, and whether these antibodies are blocking antibodies, and if this cross-reactivity translates into cross-protection.

Next to the humoral immune response, the cellular and innate immune response have been studied, albeit less extensively. Immunological studies of murine noroviruses in the mouse model indicate that both T cells and B cells are required for complete protection [92, 93].

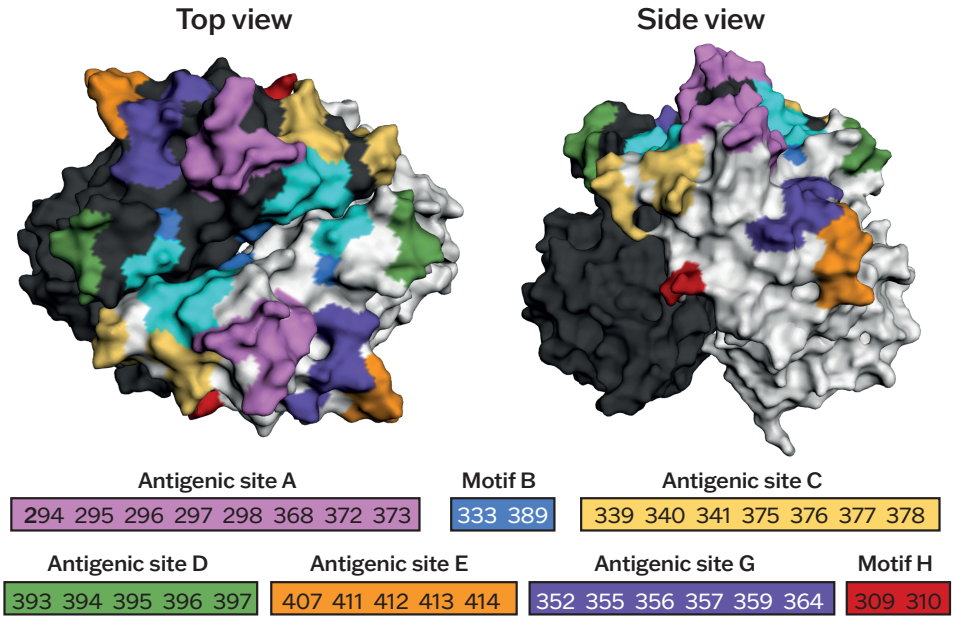


Figure 4. Antigenic epitopes and HBGA binding sites. The antigenic epitopes and HBGA binding sites are mapped onto the dimeric (subunits are in **grey** and **white**) P domain structure of GII.4 (PDB 5j3o). The antigenic epitopes were used from Tohma *et al.* [22] and the HBGA interaction sites were used from Tan *et al.* [21]. Motif B and H refer to potential but not confirmed antigenic sites.

It is not known how much of the research on murine noroviruses and the murine infection model can be extrapolated to humans. Norovirus-specific T cells have been found in the blood of adults and children but their role in protection against infection or illness is not known [75, 94-96].

In conclusion, antibodies are the most common studied component when investigating anti-norovirus immune responses. The high seroprevalence in the population indicates high exposure but the cross-reactivity between genogroups and genotypes needs to be further investigated.

Evolution

Norovirus evolution is driven by two mechanisms: the accumulation of mutations and recombination, termed antigenic drift and shift [97, 98]. Based on their evolutionary dynamics, noroviruses are categorized into static and evolving genotypes. Only GII.4 and new GII.17 lineages are considered to be evolving genotypes [4, 99] but some additional evidence also suggests the fixation of amino acid substitutions in GII.2 and GII.12 [99, 100]. The mean evolutionary rate of different norovirus genotypes is

similar, and ranges from $5.4 \times 10^{-3} - 2.2 \times 10^{-4}$ nucleotide substitutions/site/year. However, the ratio of nonsynonymous substitutions (dN) to synonymous substitutions (dS) was twice as high in GII.4 and GII.17 compared to other genotypes [99].

The predominance of GII.4 viruses has been linked to the sequential emergence of new variants in the human population replacing the previous one (**Fig. 5**) [99]. Genetic diversity is limited within variants but 8-25 amino acids substitutions can accumulate in the capsid within ~2 years resulting in a new variant [101]. Since the mid-1990s, six major norovirus GII.4 variants have been recorded to cause pandemics: Grimsby 1995 (or US95_96), Farmington Hills 2002, Hunter 2004, Den Haag 2006b, New Orleans 2009, and Sydney 2012 [102]. It is speculated that pre-1995 GII.4 strains (Camberwell-like) produced typically low-level endemic disease in human populations [103]. And that the accumulation of a series of mutations resulted in the epidemic spread of the post-1996 GII.4 strain (Lordsdale/Grimsby). Intermediate strains to fill the genetic gap between consecutive GII.4 are missing and minimal diversity has been detected within a GII.4 outbreak season. This raises the question of the origin of these strains.

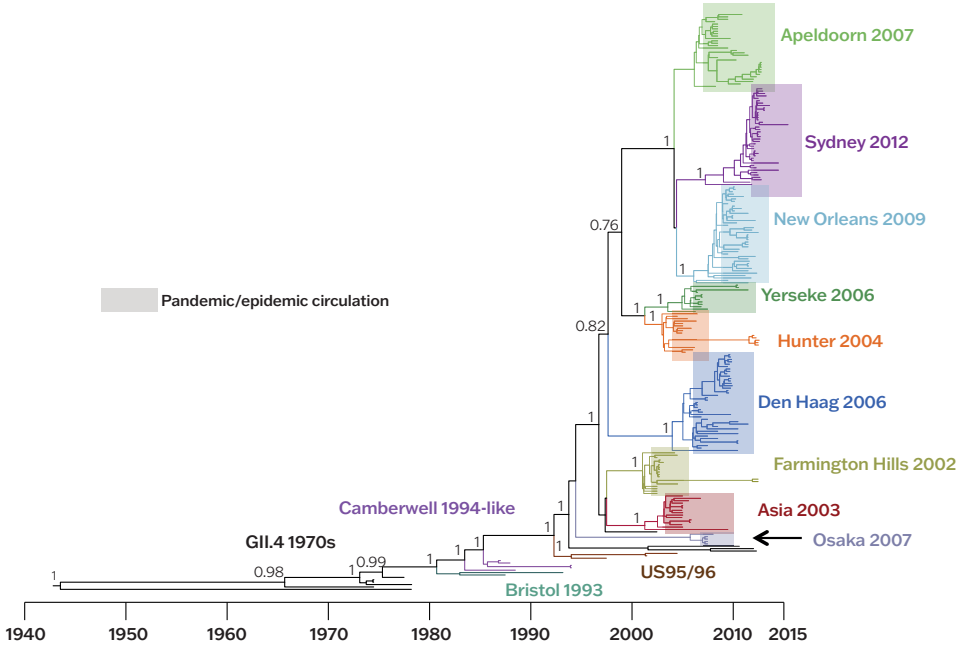


Figure 5. Epochal evolution of GII.4 variants. Temporal MCC tree of GII.4 VP1 sequences from major pandemic and epidemic variants. Posterior supports are shown on trunk nodes. Figure from Ruis *et al.* [171].

In comparison, non-GII.4 GII genotypes as well as GI genotypes are subject to less adaptive pressure and undergo only limited evolution with little genotypic innovation [4, 99]. Based on modeling of norovirus GII and GI P domain structures, Donaldson *et al.* have hypothesized that GI noroviruses have a more limited structural plasticity compared to GIIs, indicating that they have a low tolerance for changes that would enable them to escape herd immunity [4].

In addition to antigenic drift, genetic diversity is further increased by genomic recombination between viruses from either different genotypes or different variants that simultaneously infected the same cell. This occurs mostly between ORF1 and ORF2 [98, 104, 105] but the ORF2 and ORF3 junction has been reported to recombine as well [105]. The precise mechanism of recombination is not understood but one explanation would be a template switch of the RdRp. In this scenario, the RdRp would initiate positive sense RNA synthesis from the full-length negative strand and switch template to continue on the negative sense subgenomic RNA of a co-infecting virus [104]. While the emergence of new pandemic strains is usually driven by antigenic changes in the capsid, the emergence of at least four recombinants, GII.4[P31], GII.17[P17], GII.2[P16], and GII.4[P16], has been linked to the acquisition of a novel ORF1. In contrast to the capsid evolution, the role of recombination for norovirus evolution is less well studied and understood.

In conclusion, norovirus evolution is driven by the accumulation of mutations and genetic recombination. GII.4 differs from other genotypes due to its epochal evolution that is driven by changes in antigenic epitopes on the capsid. However, it has been hypothesized that the emergence of some recombinant viruses is associated with changes in the non-structural proteins rather than mutations in the capsid.

Reservoir

The origin of new variants, as well as the sporadically emerging variants and genotypes, are not known. But several potential reservoirs have been proposed.

Immunocompromised patients: In an average norovirus infection virus shedding is limited to ~2 weeks, leaving a short period for the virus to evolve and to accumulate mutations. In contrast, immunocompromised patients can shed viruses for up to several years [106-111]. This prolonged shedding as well as the limited immune pressure results in an increased virus diversity in these patients, which are mostly GII.4 variants but also other (GII.3, GII.7, and GII.17) genotypes [106, 108, 109, 112-114]. Interestingly, these sequences are genetically distinct from viruses circulating in the general population, thus indicating that these interhost variants do not contribute to newly emerging strains [107]. In addition, very little is known about the infectiousness of these evolved strains and whether they can infect healthy individuals. To date, transmission of these viruses has only been reported in nosocomial settings where

chronic shedders at an early stage of infection (first 17 days after diagnosis) had spread the virus to other patients. However, there is no evidence of these viruses being transmitted outside the hospital [107, 110, 115]. In addition, norovirus isolated from a chronic patient over several weeks was successfully cultured in the zebrafish model and the accumulated mutations did not affect viral replication [116].

Unsampled populations: Populations that are under-sampled include certain geographic regions as well as asymptomatic and sporadic cases. Between 7% and 30% of individuals are presumed to be asymptomatic depending on the setting, region, and age [117]. Only a few outbreak investigations have shown that asymptomatic shedders were involved in transmission during an outbreak [118-121]. In concordance, a study investigating five nosocomial outbreaks described that gastroenteritis cases were more frequently linked to transmission from symptomatic patients than to asymptomatic shedders [122]. Asymptomatic shedders have similar viral loads to symptomatic patients, however, they shed the virus for a shorter period (5.6 versus 9.8 days) possibly explaining, in part, the relatively small contribution to transmission compared to symptomatic patients [123-126]. It should be noted that in contrast to immunocompromised patients, prolonged shedding is less frequently reported in immunocompetent individuals and is mostly observed in birth cohort studies [40, 127-130].

Incomplete surveillance of certain geographic areas could also lead to an inadequate understanding of norovirus emergence. This is evident by the findings of several African and a Peruvian study that detected several of the pandemic GII.4 variants as well as the 2015 GII.17 years before they became the dominant variant [40, 131]. One method to study the diversity of enteric viruses in a given population, regardless of symptoms, is wastewater surveillance. Noroviruses have been detected in sewage, wastewater, and surface water globally; South Africa [132-134], Kenya [135], the Netherlands [136], Nicaragua [137], and Tunisia [138]. While sometimes the virus diversity that is found in water represent strains that are found in clinical samples in a region, some studies have found genotypes that did not notably circulate in a population [132, 135, 138]. GII and GII.4 in particular cause the majority of documented gastroenteritis outbreaks, while GI [31, 139] is less frequently found and GIV is rarely detected in patients [31, 140, 141]. The exception is Egypt where 30% of acute infections were caused by GI genotypes [41]. In contrast, GI is readily found in sewage with sometimes similar frequencies as GII strains [132-134, 139, 142] as is GIV albeit less frequently [143-146]. This discrepancy could be a result of different stabilities of genogroups/genotypes in the environment; GI viruses have been proposed to be more stable in sewage than GII [142]. Alternatively it could indicate that GI and GIV might be more prevalent in the population than previously thought, causing less severe disease or asymptomatic infections and thereby evading medical attention.

Animals have also been proposed as a possible reservoir for emerging strains of re-emerging genotypes. No animal norovirus has been found in humans, but antibodies directed against bovine and canine noroviruses have been detected in humans, suggesting some level of exposure of humans to animal noroviruses [147-150].

In conclusion, many norovirus infections likely go undetected because they occur in regions or population groups that are not sufficiently surveilled. Closing these surveillance gaps will help to understand and eventually interfere with norovirus emergence.

Prevention and treatment

Currently, the treatment of norovirus is hindered by the lack of an approved vaccine or antiviral drug, leaving prevention of norovirus transmission as the best strategy to limit outbreaks. Norovirus transmission is primarily prevented by proper hand hygiene and disinfection of surfaces, as well as isolation of positively tested people in healthcare settings [151-153]. Because alcohol-based hand hygiene has little effect, handwashing with soap is recommended instead [153, 154]. Noroviruses are very stable on surfaces and cleaning contaminated surfaces with detergent or ethanol is not sufficient [155]. A first round of detergent followed by a second round of hypochlorite/chlorine treatment is most efficient [155, 156].

To date, no vaccine is approved but several candidates are in preclinical and clinical trials. Ongoing vaccine trials focus on recombinant vaccines as no system is in place to test inactivated or live attenuated vaccines. Currently, three vaccine types are in development: non-replicating virus-like particles (VLPs), P particles, and recombinant adenoviruses (reviewed in [157]). One vaccine is in phase II trials, a bivalent VLPs based vaccine containing GI.1 and three GII.4 variants from Takeda. In preclinical studies, the vaccine was highly immunogenic and elicited an antibody response that also reacted against GI.1, GII.1, GII.3, and GIV [158]. In a randomized, double-blind, placebo-controlled trial healthy adults were vaccinated and subsequently challenged. While the disease severity was decreased, the overall prevalence of norovirus illness was not significantly reduced [90]. Further phase II trials are ongoing.

Due to the historical lack of a robust cell culture system and a small animal model, the search for antivirals against noroviruses has been delayed (reviewed in [159]). But recently the development of two culture systems has opened the doors for *in vitro* testing of antiviral therapeutics. The RdRp is an attractive target as there is no host homolog and off-target effects are therefore minimal. RdRp-targeting drugs 2'-C-methylcytidine (2CMC), favipiravir, and ribavirin are nucleoside analogs that induce chain termination or increase the mutation rate leading to lethal mutagenesis. Even though none of these drugs are currently approved for norovirus infections, ribavirin, favipiravir, and nitazoxanide have been administered off-label to norovirus

patients with varying effects [160-163]. The only antiviral that has progressed to clinical trials is nitazoxanide, for which the mechanism of action is unknown. In a phase II randomized double-blind trial, nitazoxanide administration reduced the duration of gastrointestinal symptoms from 2.5 to 1.5 days [164].

Most efficient antivirals that are approved limit infection of chronic diseases such as hepatitis B and C viruses, herpesviruses, and human deficiency virus [165]. Due to the fast onset of illness for norovirus, it is questionable if antivirals will be able to prevent disease or if their application will be restricted to the reduction of disease burden in chronically infected patients.

As an alternative treatment, oral human immunoglobulins have been administered to some chronic norovirus shedders. One study reported successful treatment in 11/12 patients and another case report noted resolution of symptoms 48h after treatment [166, 167]. With regards to norovirus elicited immunity in immunocompromised patients, only two studies have investigated antibody response upon infection. In a study investigating 10 patients, the antibody response was detected after confirmed infections with GII.1 or GII.4 [168] and in one case study of a patient undergoing chemotherapy, blocking antibodies were correlated with a decrease in viral load [169].

In conclusion, antivirals have the advantage that they target enzymes that have functional domains which are conserved across virus families, and finding an effective antiviral against noroviruses might be less restricted by norovirus diversity than vaccine development. Vaccine development, in contrast, has mainly focused on GII.4 and GI.1 since GII.4 has been the dominant genotype for several decades. In recent years, however, several non-GII.4 genotypes have emerged raising the question if other genotypes should be considered as well.

Scope of the thesis

This thesis aims to obtain a better understanding of the emergence of new norovirus variants and recombinants as well as the re-emergence of previously circulating noroviruses. We investigated possible reservoirs in which these viruses could circulate and emerge from as well as possible mechanisms contributing to the emergence of new recombinants.

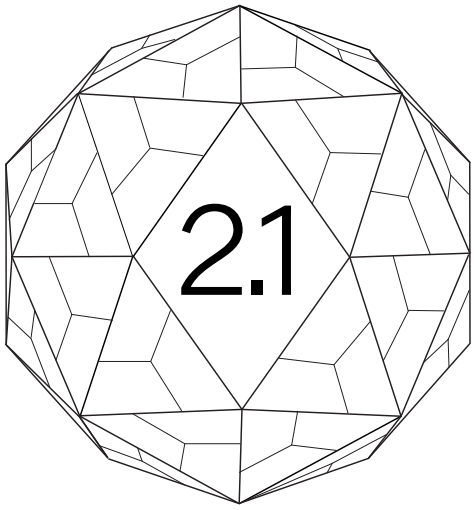
In **Chapter 2**, we focused on the human-animal interface. In the systematic review (**Chapter 2.1**) we have summarized all published sequencing and serology data on norovirus transmission between humans and animals and we discuss the potential of noroviruses to jump the species barrier. The detection of human norovirus sequences in stool samples of domestic and wild animals shows that there is at least human-to-animal transmission. In **Chapter 2.2**, we have performed phylogenetic analyses to investigate the evolutionary relationship between human noroviruses circulating in the human population and those detected in animals to assess the likelihood of long-term circulation of human noroviruses in animals. There is a broad range of animal species that harbor their own types of norovirus and we further assessed these animals as possible reservoirs for emerging noroviruses in **Chapter 2.3**. We analyzed the presence and distribution of the known susceptibility factor in humans, the histo-blood group antigens (HBGA), on intestinal tissue as well as the capacity of human noroviruses to bind to animal tissue. We show that a broad range of animals expresses HBGAs and that conversely norovirus attachment was detected to almost all species. This implies that several animal species could be susceptible to human noroviruses.

In **Chapter 3**, we focused on the role of humans in norovirus circulation and emergence. Due to their lack of a fully developed immune system, children have been hypothesized to act as a vessel for norovirus diversity. Using serology, we investigated the norovirus diversity circulating in children and showed that primary infections are predominantly caused by the most commonly detected norovirus genotypes (**Chapter 3.1**). A second hypothesis proposes that norovirus diversity could originate in immunocompromised patients. Due to their not fully developed immune system and the inability to clear norovirus infections efficiently, immunocompromised patients suffer from prolonged infections lasting up to years. During this long-term circulation under reduced or no immune pressure, intrahost evolution is higher than in immunocompetent hosts. This results in norovirus strains that are genetically distant from commonly circulating strains. In **Chapter 3.2**, we addressed the effect of intrahost evolution in immunocompromised patients and the consequential implications for treatment.

In **Chapter 4**, we investigated the role of the polymerase in norovirus emergence. While new GII.4 variants commonly are associated with amino acid substitutions in the capsid, some pandemic strains have obtained a new ORF1 sequence. Here, we investigated how recombination could contribute to virus emergence.

In addition to intra- and interhost virus evolution and host immunity, virus transmission is an important aspect of understanding virus dynamics and epidemiology. In **Chapter 5**, we reviewed the recent advancements in model systems and methods to study norovirus transmission experimentally.

In **Chapter 6**, we have summarized the findings of the thesis and discussed them in light of more recent norovirus insights.



Human norovirus at the human-animal interface

Animals as reservoir for human norovirus

Nele Villabruna, Marion P.G. Koopmans and Miranda de Graaf

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Abstract: Norovirus is the most common cause of non-bacterial gastroenteritis and is a burden worldwide. The increasing norovirus diversity is currently categorized into at least 10 genogroups which are further classified into more than 40 genotypes. In addition to humans, norovirus can infect a broad range of hosts including livestock, pets, and wild animals, e.g., marine mammals and bats. Little is known about norovirus infections in most non-human hosts, but the close genetic relatedness between some animal and human noroviruses coupled with lack of understanding where newly appearing human norovirus genotypes and variants are emerging from has led to the hypothesis that norovirus may not be host restricted and might be able to jump the species barrier. We have systematically reviewed the literature to describe the diversity, prevalence, and geographic distribution of noroviruses found in animals, and the pathology associated with infection. We further discuss the evidence that exists for or against interspecies transmission including surveillance data and data from in vitro and in vivo experiments.

1. Introduction

The majority of emerging infectious diseases that affect humans originate from animal reservoirs, predominantly wild life, including bats, rodents and birds. Norovirus is one of five genera of the family *Caliciviridae* and the most common non-bacterial cause of foodborne gastroenteritis worldwide. Noroviruses are currently categorized into at least seven genogroups (GI-GVII) that are further divided into more than 40 genotypes [172]. The virus contains three open reading frames (ORFs), ORF1 encoding the polyprotein that includes the viral polymerase, and ORF2 and ORF3 encoding the major- and minor capsid protein (VP1, VP1), respectively [173]. Recombination between ORF1 and ORF2 frequently occurs and therefore a dual nomenclature describing both the polymerase and capsid genotype is used [98, 104, 174]. Viruses from genogroups GI, GII and GIV are known to infect humans. Animal noroviruses including viruses found in pigs, dogs, and cats are closely related to human strains and cluster within GII (porcine norovirus) and GIV (feline and canine norovirus), respectively [172]. Noroviruses belonging to the other genogroups infect a broad range of hosts that includes livestock animals such as cows and sheep but also marine mammals and rodents. In the past years, an increasing number of metagenomic studies have led to the discovery of additional noroviruses in new animal hosts and it seems evident that we lack understanding of the full diversity of noroviruses and their host range [175, 176]. Most human infections and outbreaks are caused by viruses belonging to GI and GII. The GII.4 genotype viruses have been particularly prevalent in the past two decades, and evolve through accumulation of mutations but also by recombination. Such recombinants and other new genotypes emerge regularly but the origin of these new viruses is not well understood [177]. This regular detection of novel strains and the reporting of human-like norovirus genotypes in stool samples of symptomatic and

asymptomatic farm animals have sparked interest in the possible role of animals as potential zoonotic reservoir for these emerging strains [178-181]. Antibodies directed against bovine and canine norovirus have been detected in humans suggesting some level of exposure of humans to animal norovirus [147-150]. For other viruses of the *Caliciviridae* family, interspecies transmission has been reported including some case reports of zoonotic events between marine mammals and humans (reviewed in [182]).

This systematic review summarizes the literature on the known animal reservoir for norovirus, the virus diversity, prevalence, and geographic distribution, as well as pathological findings associated with norovirus infections in animals. We will further discuss the existing evidence and probability of interspecies transmission including susceptibility of animals used as models in norovirus research. There are several reviews that focus exclusively on the role of mice in norovirus research [183-185]; therefore, we will discuss murine norovirus only in context of surveillance of wild animals. Molluscs are an important vehicle of foodborne norovirus transmission, but do not support norovirus replication and have been reviewed elsewhere [186, 187].

2. Results

2.1. Search output

The search yielded 6702 papers of which a total of 182 were included in the review. An additional nine papers were later included (see methods).

2.2. Noroviruses in domesticated and wild animals

Norovirus was first described from a gastroenteritis outbreak in 1968, which affected children in a school in Norwalk, Ohio, USA [188]. In 1972, the virus was visualized for the first time by immune electron microscopy revealing "small round structured viruses" (SRSV) of 27–35 nm in diameter, which was used as their first classification [189]. Viruses of similar morphology were soon described from stool samples of domestic calves and pigs, and sequencing confirmed the presence of viruses belonging to the same family as human noroviruses. To date, porcine noroviruses are genetically most similar to human norovirus; porcine noroviruses have been classified among a diverse range of human norovirus genotypes in GII as GII.11 (prototype SW918), GII.18 and GII.19 [179, 190] and have been found in stools and intestinal content of pigs all over Europe, North and South America, and Asia (**Fig. 1A** and **B**, **Table 1**).

In most countries, the overall detection rate of porcine norovirus in stool samples is low (0%-16.6%) and outbreaks have not been reported, although there is evidence for symptomatic porcine norovirus infections. When specific-pathogen-free (SPF) piglets were inoculated with GII.11 or GII.18 positive fecal filtrate they showed mild to moderate diarrhea within 1 day post inoculation (dpi) and norovirus RNA

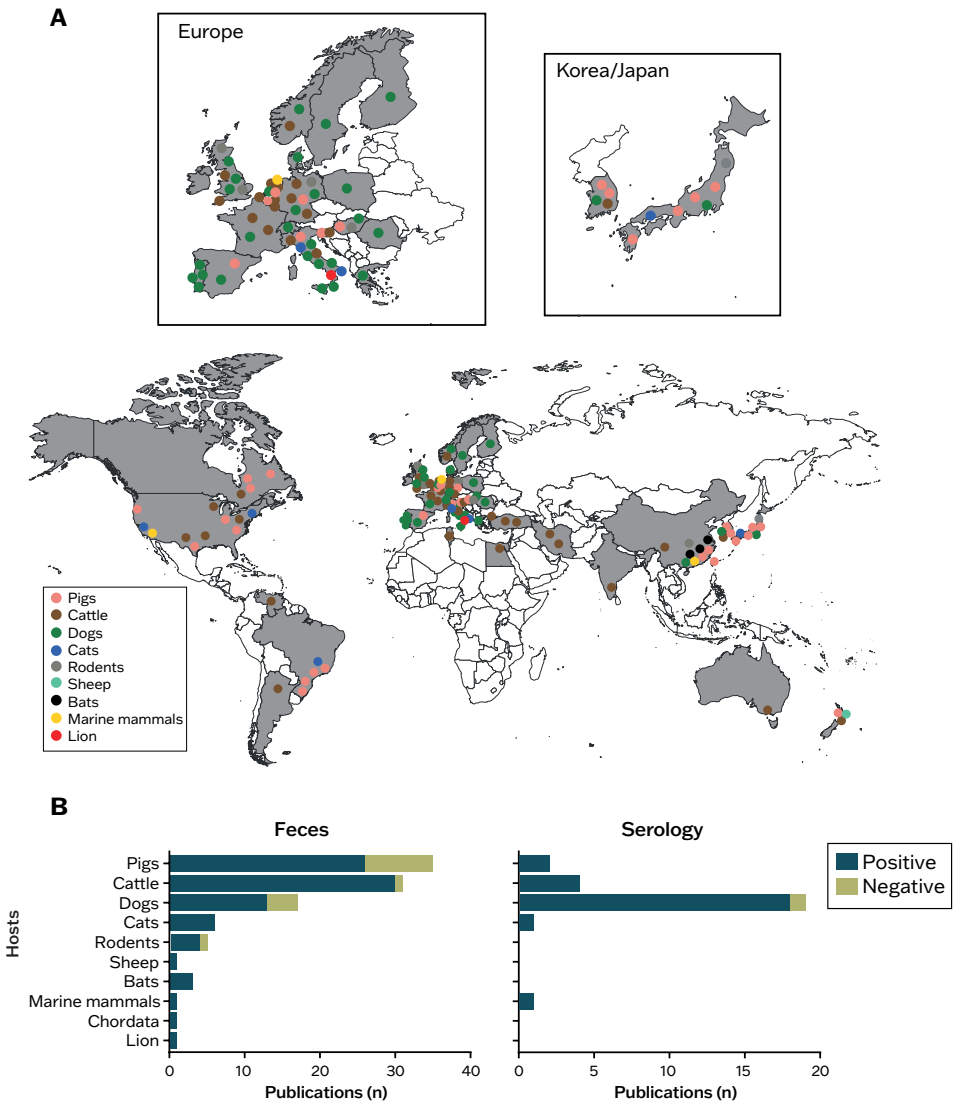

Figure 1. Studies describing the presence of animal norovirus across the world. (**A**) Countries in which animal norovirus have been detected are colored green. Each dot represents a study and location where animals have been found positive by either RT-PCR, real-time RT-PCR, or serology. The color indicates the host. (**B**) Number (n) of publications reporting positive versus negative surveillance results in different hosts for PCR results in feces and serology studies. Note that a paper that studied GVI.2 seropositivity in dogs in 14 European countries is listed as 14 studies in 1B [238]. Details of the studies are listed in **Table 1** and **Table S1**.

was amplified from intestinal content as well as from sera [191, 192]. The majority of surveillance studies have been screening healthy pigs from farms and slaughterhouses [178, 179, 190, 193-209]. Asymptomatic finisher pigs most commonly tested positive, but porcine noroviruses have also been found in stools from asymptomatic pigs of other age categories as well as diarrheic piglets [191, 199, 210]. Virus circulation is thought to be widespread. A survey of pigs found antibodies to GII.11 virus like particles (VLPs) in 71% and 36% of pigs in the USA and Japan [211].

The SRSV found in stool samples from cattle have subsequently been characterized as bovine norovirus GIII.1 (Jena agent) and GIII.2 (Newbury Agent 2), discovered in cattle in Germany and England, respectively [181, 212]. Upon experimental inoculation with a GIII.1 or GIII.2 gnotobiotic calves develop diarrhea, shed virus for several days and seroconvert, although not in 100% of the cases [55, 61, 180, 213-218]. Both genotypes are widely distributed among diarrheic and healthy cattle, juveniles, and adults, although GIII.2 viruses have been found more frequently than GIII.1. The majority of published surveys has tested diarrheic calves, in which bovine norovirus was frequently found [147, 207, 215, 219-232]. One case–control study that investigated pathogens associated with calf diarrhea in the USA tested 444 samples of 1-2-week-old diarrheic and asymptomatic calves for a panel of 11 enteric pathogens (bacteria and viruses) using real-time RT-PCR with bovine norovirus specific probes. A prevalence of 44.7% was reported in diarrheic and 16.3% in healthy calves [219]. Less is known about bovine norovirus in adult cattle. One study compared prevalence of bovine norovirus RNA in pooled manure samples of 75 dairy farms with those of 43 veal calf farms. A high proportion (44%) from the veal calf farms was positive, but bovine norovirus RNA was not detected in samples from the dairy farms [178]. The prevalence of antibodies to GIII.1 or GIII.2 VLPs was >70%, independent of location (**Table 1**) and only very few studies failed to detect GIII viral RNA or antibodies (**Fig. 1B**). A proposed third GIII genotype, GIII.3, was found in asymptomatic sheep in New Zealand [203].

While pigs and cows are the best studied non-human hosts–apart from mice–noroviruses have also been detected in stool samples from cats and dogs. Both animal species were shown to be infected by viruses belonging to genotype GIV.2, while dogs are also hosts of canine GVI and GVII strains. The first carnivore norovirus was documented in a captive lion cub (*Panthera leo*) in Italy that had died of severe hemorrhagic enteritis [233]. This new strain shared ~70% aa VP1 identity with the human GIV.1 sequence, which is only identified sporadically in the human population, but is more commonly detected in sewage samples [145]. One outbreak study documented the arrival of two diarrheic young dogs into a kennel in Lisbon [234]. Two days later, five young dogs housed in the same kennel developed diarrhea and the isolated GVI.2 sequences were identical to each other.

Table 1. Summary of studies detecting animal norovirus in animals, either in feces or by serology. Details of each study can be found in **Table S1**.

Location	Host	Genotype	Prevalence in % (References)	
			Serology	Feces
The Americas				
USA	Pigs	GII.18, GII.11, GII.19	71 [211]	0-19 [190, 193, 211, 296]
	Cattle	GIII.1, GIII.2	100 [297]	29-72 [219, 220, 298]
	Cats	GIV.2		17-43 [240, 244]
	Sea lion	GII/GIV		9 [247]
Canada	Pigs	GII, GII.11, GII.18		2-85 [195-197]
	Cattle	GIII.2		1 [195]
Venezuela	Pigs	all		0 [204]
	Cattle	GIII		0.7 [299]
Argentina	Cattle	GIII.1, GIII.2		3 [300]
Brazil	Pigs	GII.11, GII.18, GII.19		0-52 [209, 301-304]
	Cats	GIV,2		3 [243]
Asia/New Zealand/Australia				
China	Pigs	GII.11, GII.18, GII.19		0-17 [191, 192, 198, 200]
	Cattle	GIII.1		11 [229]
	Bats	NA		3-4 [175, 250]
Taiwan	Pigs	GII.11		1.6 [199]
Japan	Pigs	GII.11	36 [211]	0.4-15 [179, 201, 210]
	Dogs	GIV		2 [242]
	Cats	GIV.2		1.2 [242]
	Rodents	GV		0-14 [305]
Korea	Pigs	GII.11, GII.18		0.5-2 [202, 306]
	Dogs	Canine norovirus	16 [307]	3 [307]
	Cattle	GIII.1, GIII.2		9 [230]
Iran	Cattle	GIII.1, GIII.2		18-40 [231, 308]
Turkey	Cattle	GIII.2		4-9 [232, 309]
India	Cattle	GIII.1		0.4 [282]
New Zealand	Pigs	GII.11		9 [203]
	Cattle	GIII.1		54 [310]
	Sheep	GIII.3		24 [203]
Australia	Cattle	GIII.2		25 [187]
Europe				
Italy	Pigs	GII.11		0-0.5 [288, 311]
	Cattle	GIII.1, GIII.2		11-21 [221, 221]
	Dogs	GIV, GVI	5-60 [238, 239, 254]	2-5 [236, 312]
	Lion	GIV.2		100 [233]
	Cats	GIV.2	16 [241]	3 [239]
Spain	Pigs	all		12 [313]
	Dogs	GVI		8 [236]
Portugal	Dogs	GIV, GVI	64 [238]	23-28 [234, 235, 237]
Greece	Dogs	GIV.2		8 [3143]
France	Cattle	GIII.1, GIII.2		20-37 [225, 226]
	Dogs	GVI.2	20 [236, 238]	0 [236]
Switzerland	Dogs	GVI.2	20 [238]	
Germany	Pigs	GII.18		14 [206]
	Cattle	GIII.1, GIII.2	66-99 [315, 316]	93 [317]
	Dogs	GIV, GVI.2	16 [238]	4 [236]
	Rodents	GV		10 [318]
Netherlands	Pigs	GII.11		2 [178]
	Cattle	GIII.2	0-44 [178]	4 [319]
	Dogs	GVI.2	34 [238]	
	Porpoise	not classified yet	24 [62]	10 [62]
Belgium	Pigs	GII.19		4.6 [287]
	Cattle	GIII.2	93 [223]	4-9 [223, 224, 254, 320]

UK	Cattle	GIII.1, GIII.2	66-98 [217, 315]	11 [321]
	Dogs	GIV, GVI, GVII	45-48 [238, 255, 322]	0 [255]
	Rodents	GV		22-67 [323]
Ireland	Pigs	none		0 [205]
	Dogs	none	0 [238]	
Denmark	Dogs	GVI.2	20 [238]	
	Rodents	none		0 [267]
Sweden	Dogs	GVI.2	40 [238]	
Norway	Cattle	GIII.1, GIII.2		50 [215]
	Dogs	GVI.2	32 [238]	
Finland	Dogs	GVI.2	70 [238]	0 [253]
	Rodents	none		0 [266]
Poland	Dogs	GIV.2	32 [238]	
Slovenia	Pigs	GII.11, GII.18		1.2 [207]
	Cattle	GIII.2		2 [207]
Hungary	Pigs	GII.11		6 [324]
	Dogs	GVI	0 [238]	3 [325]
	Rodents	GV		24-67 [326]
Africa				
Egypt	Cattle	GIII.2		24 [227]
Tunisia	Cattle	GIII.2		17 [228]
South Africa	Pigs	none		0 [256]
Ethiopia	Pigs	GII.1		0 [208]

Canine noroviruses sequences have since been detected in feces from healthy and sick dogs from kennels, shelters, and households in South America, Europe, and Asia (**Fig. 1** and **Table 1**). To date, no infection studies have been conducted with canines and the pathology of noroviruses in dogs is therefore unclear. However, during a study in Portugal, canine norovirus RNA was found more often in the stool samples of symptomatic dogs compared to asymptomatic dogs (40% versus 9%), suggesting they play an important role as cause of disease [31, 235]. In a Europe-wide study, an overall 4.4% prevalence was found for diarrheic dogs while none of the healthy animals tested positive [236]. A strong seasonal pattern was observed during a four-year period of sampling dogs in Portugal, with the highest prevalence (36%) in winter and lowest (7%) in autumn, similar to the seasonality observed for norovirus in humans [31, 237]. A serological survey screening dogs from 14 different countries found variable prevalences of antibodies to GVI.2, ranging from 0% in Hungary and Ireland up to 60% in Portugal [238].

The first evidence for feline noroviruses was provided through an Italian study, where 16% of cats tested positive for GIV.2-specific antibodies, with the highest prevalence among stray cats (32%) [239]. Three years later, in 2012, a feline norovirus was discovered during a gastroenteritis outbreak in cats in a shelter in the USA [240]. The cats were negative for known feline parasites, but a full norovirus genome was recovered (JF781268). Similar viruses were later detected, mostly in diarrheic cats [241-244]. After inoculation of SPF cats with feline norovirus, the cats shed the virus up to 7 dpi, viral RNA was detected in sera of all cats, three of the four cats developed

diarrhea and one started vomiting [245]. Another study using the same inoculum showed that cats developed IgG against recombinant VP1 protein identical to the strain used for the experimental infections [246].

Apart from domesticated animals, noroviruses have also been detected in wild animals, such as harbor porpoise (*Phocoena phocoena*) and californian sea lions (*Zalophus californianus*) [62, 247]. Neither of these viruses could be assigned to an existing genogroup. Further investigation found 10% of harbor porpoise intestinal tissues RT-PCR positive and 24% of the animals seropositive for porpoise norovirus, suggesting that norovirus infections are common infection in these animals. With the recently increasing trend of metagenomic studies, additional noroviruses have been identified. In a metagenomics analysis of bats intended to decipher the bat virome, a full norovirus genome was recovered from intestinal tissue of *Rhinolophus pusillus* bats captured in two Chinese provinces [175]. In one location the prevalence in fecal samples was as high as 20%. This strain belongs to a new genotype which shares highest sequence homology with GV norovirus (**Fig. 2**) [248]. Subsequent studies have detected norovirus in two species of insectivorous bats in China, namely *Rhinolophus sinicus* and *Rhinolophus affinis* [249, 250]. Most of the animal noroviruses have not been detected in animals other than the species they were first identified in. Exceptions are the GV noroviruses, which are detected in mice and rats, and the canine/feline GIV and GVI noroviruses.

2.3. Is there evidence for cross species transmission?

Since the first norovirus has been detected from animals, the question has been raised whether norovirus can jump the species barrier. To date, there are no controlled outbreak studies during which both animals and humans have been sampled simultaneously. One calicivirus outbreak in a nursing home in 1983 in the UK was epidemiologically linked to a sick dog. While virus particles were found in the patients, no stool sample was available from the dog and only antibodies against the same virus could be detected [251].

2.3.1. Animal-to-human transmission

To date, no animal noroviruses have been detected in human stool, but some serological evidence hints to possible transmission from animals to humans. This includes a handful of studies that reported seroprevalence against bovine [147, 148, 252] and canine [149, 150] norovirus in humans. A Dutch study compared antibody titres against GIII.2 VLPs from 210 bovine or porcine veterinary specialists against age, sex, and residence matched controls with the aim to evaluate whether higher exposure to animals is reflected in increased titers against these animal noroviruses [252]. More veterinarians had anti-GIII.2 IgG antibodies compared to the control group (28% versus 20%).

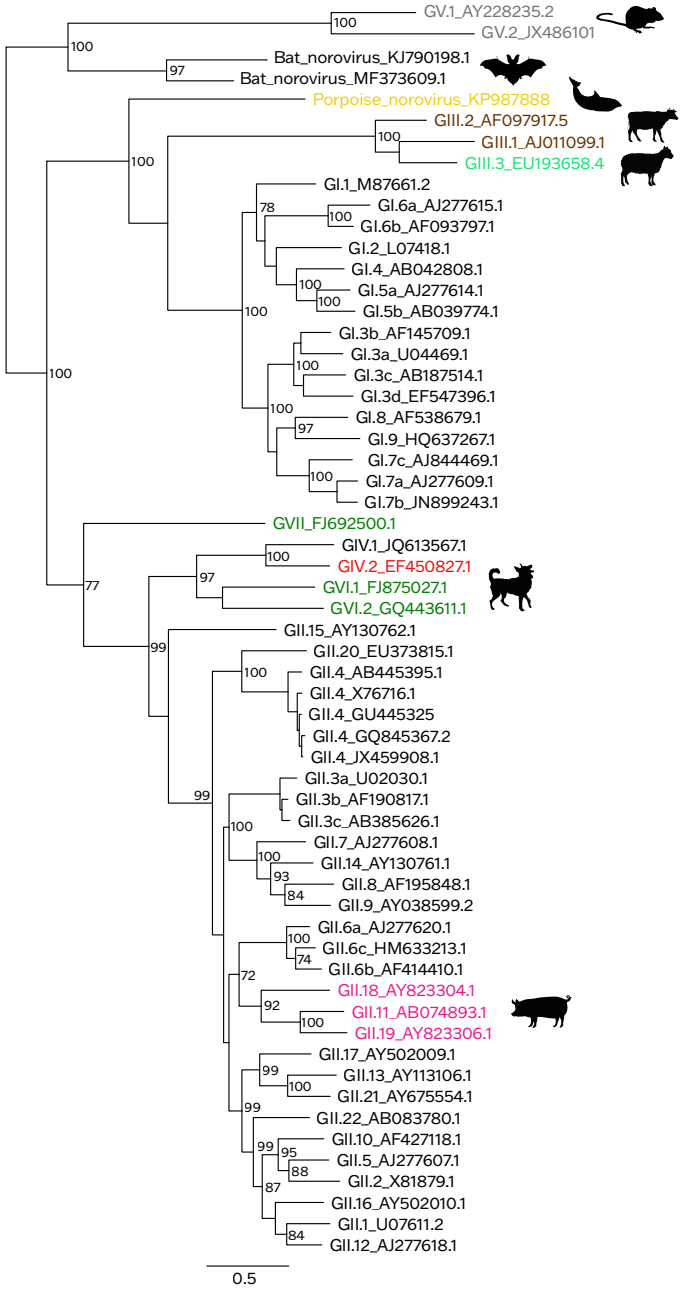

Figure 2. Maximum-likelihood tree of open reading frame 2 (ORF2). The tree was inferred by PhyML 3.0 software (http://www.atgc-montpellier.fr/phyml/) by using the general time reversible nucleotide substitution model. Bootstrap values >70 are shown. Scale bars indicate nucleotide substitutions per site. Animal noroviruses are colored with same color code as in **Fig. 1A**.

Similarly, the seroprevalence of antibodies to canine GVI.2 norovirus VLPs was tested in a cohort of 373 veterinarians versus age, sex, and district matched controls. Of the veterinarians, 22.3% were seropositive for GVI.2 in comparison to 5.8% in the control group [150]. Anti-GIII antibodies were also detected in 26.7% of adult blood donors in Sweden [148] and in a birth cohort in India, which compared seroprevalence of mothers and their children [147]. However, the possible presence of cross-reactive antibodies needs to be considered in these studies: the GIII.2 response was in part correlated with GI.1 response, but not with the GII.4 response. The finding that some sera contained higher antibody titers against GIII.2 than human norovirus indicates that not all anti-GIII.2 response can be explained by cross-reactivity [252]. Importantly, no cross-reactivity between bovine GIII.2 and human GI.3, GII.1, GII.3, GII.4, GII.6 was detected when convalescent anti-GIII.2 sera of a gnotobiotic calf or specific anti-GIII.2 or GII.3 antibodies were used [148, 214]. Cross-reactivity between GVI.2 and GII.4 was assessed by preincubating GVI.2 positive sera with GVI.2 VLPs before assessing their binding to GII.4 or GVI.2. Preincubation with GVI.2 blocked binding to GVI.2 VLPs but had no effect on sera binding to GII.4, suggesting that these two genotypes share no conserved epitopes [150]. In contrast, cross-reactivity was observed between more closely related human GIV.1 and canine GIV.2 noroviruses in an age stratified cohort of 535 people in Italy [149], where 28.2% of the sera reacted to both GIV.1 and GIV.2 VLPs and only 0.9% detected exclusively GIV.2 VLPs.

2.3.2. Human-to-animal transmission

Numerous studies have investigated the possibility of human norovirus transmission to animals by screening animal stool samples for human noroviruses or by investigating the seroprevalence against human norovirus strains (**Fig. 3** and **Table S2**). The closest to an outbreak study was one case-control study that included 92 dogs from Finnish households. The main inclusion criterion was that either the dog or a human in the household had suffered from vomiting or diarrhea [253]. Four dogs tested PCR positive and they all came from households in which at least two people suffered from severe gastroenteritis symptoms that had disappeared not longer than three days before the dog samples were taken. Based on a ~370 nt region two GII.4 variants and one GII.12 genotype were identified, of which one GII.4 was identical to the virus found in the owner's feces. The other strains were >98% nt identical to circulating human norovirus strains. Antibodies against GII.4 and GI.1 VLPs have been detected in dogs sampled in a European study and against GII.4 and GIV.1 in dogs in Italy [238, 254]. Both studies found that sera from some animals reacted exclusively to the human strains but not to canine GVI.2 VLPs. Caddy *et al.* investigated the seroprevalence against human noroviruses (GI.1, GI.2, GI.3, GII.3, GII.4, GII.6, GII.12) in two dog populations; sera from dogs in a rehoming kennel in 1999-2001 and sera collected in 2012-2013 from a diagnostic lab. Overall, seropositivity against GI was very low, but

10.7%-18.6% were seropositive against GII VLPs [255]. The majority of seropositive dogs had antibodies detecting GII.4 viruses which was the most prevalent human norovirus circulating during this time. Only weak cross-reactivity was observed with canine sera or polyclonal sera specific for GII.4 or GVI.1/GIV.2 [255]. Combined, these studies suggest that human noroviruses could infect dogs, although more work is needed to unravel potential cross-reactivity with non-human viruses, like GVI.2 [254].

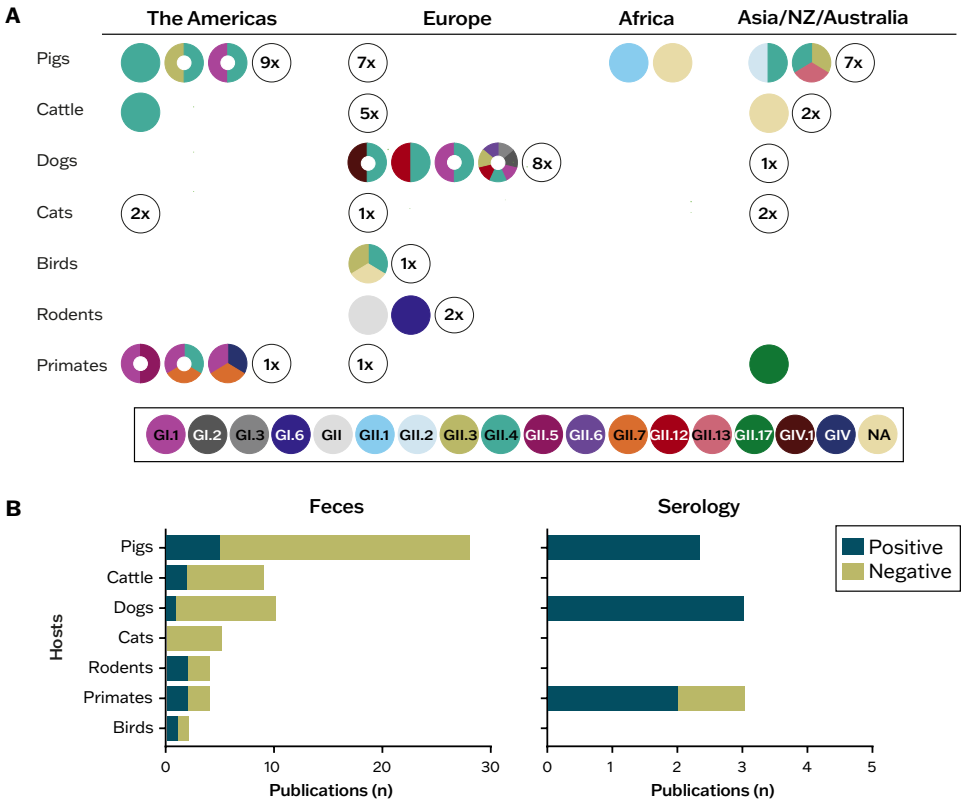

Figure 3. Human norovirus genogroups and genotypes detected in studies investigating human-to-animal transmission. (**A**) Studies that analyzed fecal samples for human norovirus sequences by RT-PCR, real-time RT-PCR or serological studies. Every circle represents one study and colors represent different norovirus strains identified through sequencing. Serological studies are marked with a central white circle, and colors here represent antigens used for the serological testing. Numbers in empty circles indicate the number of studies in which no evidence for human norovirus infection was found. NA stands for studies where the genogroup or genotype was not identified. (**B**) Number (n) of virological and serological studies of norovirus in different hosts, grouped according to results (positive versus negative). More details can be found in **Table S2**. NZ=New Zealand.

Several surveys in pigs reported human norovirus in pig feces and two reported more than one genotype [195, 199, 201, 208, 256]. In a longitudinal study in Japan intestinal content of 20 apparently healthy 6-months-old pigs were screened each month with calicivirus-specific primers. Of these, 11/354 were positive for human GII without a seasonal pattern being recognized [201]. Based on partial capsid sequences these strains were classified as GII.4, GII.3 and one GII.13, all three genotypes that had been reported in outbreaks in humans during that season. Another study tested 530 fecal samples of asymptomatic pigs (<8 month) from six farms in Taiwan, 7% tested positive with RdRp-specific primers, while GII capsid-specific primers resulted in 32% positive samples, 41% in winter and 26% in summer [199]. The GII.4 and GII.2 classified sequences were found in pigs of all age categories and from different farms. Sequences of GII.1 and GII.4 noroviruses have also been detected in feces of two healthy sows in Ethiopia and GII.4 in pig feces from two different farms in Canada [195, 208].

Antibodies recognizing human norovirus have been detected in healthy household pigs in Nicaragua and US pigs with prevalence ranging from 52%-70% [211, 257]. While those antibodies recognized VLPs of GI.1, GII.1, GII.3 and GII.4 they were not able to block their binding to pig mucin [257]. Cross-reactivity was also investigated and antibodies against GII.1 and GII.3 but not against GI genotypes cross reacted with porcine GII.11 [211]. The studies thus far raise the question if certain norovirus genotypes considered to be "human" noroviruses co-circulate among pigs. As these observations are not consistent, this could be restricted to some regions where opportunity for contact of pigs with human is higher.

During the 2014-2015 epidemic season, GII.17 was the dominant human norovirus genotype in some Asian countries [258, 259]. 32 of 50 rhesus macaques on a Chinese farm tested positive using GII.17 specific primers and a whole GII.17 genome (KX356908) was recovered from one animal [260, 261]. This GII.17 genotype was 99% identical to a human GII.17 recently detected in China [261]. Rectal swabs of juvenile rhesus macaques from a primate research center in the USA were screened by real-time RT-PCR for GI, GII, and GIV noroviruses; of the 500 samples, 8.2% were positive [262, 263]. Sanger sequencing showed that the animals were positive for 30 GI.1 and eight GII.7 strains, and yielded two full ORFs of GI.1 and GII.7 sequences (KT943503-KT943505). Surprisingly, the GI.1 sequences were not only identical to each other but also to the prototype Norwalk virus described in 1968. The GII.7 sequences were 99%-100% identical to each other and 95% identical to a human norovirus (KJ196295). Furthermore, antibodies against various human norovirus genotypes were detected in captive primates in the US; IgG against GI.1, GII.4, GII.5 and GII.7 VLPs were detected in mangabeys (85%), macaques (~60%-65%), and chimpanzees (92%) [264, 265].

Compared to surveillance in livestock animals only a few studies have investigated wild animals. Bird feces were collected during three winters (2009-2011) from fresh snow of a household waste dumping site in Finland and analyzed by GI and GII specific real-time RT-PCR [266]. Of the 115 avian feces tested, six were positive for GI and 25 for GII, albeit with high Ct values, the lowest being 36. Sequencing and typing was successful for four GII.4 (GII.4 2006a/b, 2009) and two GII.3 viruses, all at least 94% identical to known human strains. Based on cytrochrome c oxidase I sequencing, the positive feces could be assigned to gulls and crows. Another human norovirus was found in the intestinal content of a dead Norway rat that had been trapped in the sewer system in Copenhagen; a ~4000 bp sequence was recovered and was typed as a GI.Pb-GI.6 strain [267]. The virus titer was calculated to be 5×10^7 genome copies/g feces and norovirus particles were detected in feces by immunogold electron microscopy [267].

2.3.3. Susceptibility of animals to human norovirus strains

In addition to finding human norovirus in animal stool samples, noroviruses have been found to cross the species barrier under experimental conditions. To date, seven animal models have been developed to study human norovirus infection; gnotobiotic calves and pigs, immunocompromised BALB/c Rag$^{-\gamma c-}$deficient mice, Yucatan miniature pig, and three non-human primates, namely chimpanzees, rhesus, and pigtail macaques (**Table 2**). In contrast, common marmosets, cotton top tamarin, immunocompromised ferrets, and cynomolgus macaques were not found to be susceptible to infection, although only a limited number of norovirus genotypes was tested [6, 268]. All models support viral replication evident by viral shedding and seroconversion upon oral or intragastric inoculation with a high viral dose (10^4–10^6 genomes). Whereas pigs and calves developed diarrhea, both chimpanzees and rhesus macaques did not display any gastrointestinal symptoms. Virus replication was usually found to be restricted to sites of the small intestine. In mice, viral genomes could be amplified from various organs, and in minipiglets, low levels of the virus were additionally found in blood as well as in tonsils, spleen, and lymph nodes [56, 58]. Pathological changes were detectable only in calves and pigs but not in primates. These changes included villous blunting, atrophy, and an increase in inflammatory cells in the lamina propria. Norovirus antigen was detected in the small intestine, varying between duodenum, jejunum, and ileum depending on the animal and the virus strain used for inoculation. Noteworthy, in pigs as well as in chimpanzee experiments, animals were chosen based on their histo-blood group antigen (HBGA) and secretor status. In pigs, take of infection was strongly dependent on their HBGA phenotype and secretor status. HBGA type A+/H+ pigs were more readily infected than type A-/H- pigs [79]. However, while two culturing systems have been described for human norovirus [63, 65], attempts to grow human or animal norovirus in animal cell culture have not yet been successful [269, 270].

Table 2. Summary of animal models for human norovirus.

	Gnotobiotic calf [55]	**Gnotobiotic pig** [53, 54, 327-334]	**Mini piglet** [56]	**Rhesus macaques** [260, 268, 335]	**Pigtail macaques** [336]	**Chimpanzee** [57, 337]	**Mice Balb/c RAG/$\gamma c^{-/-}$ Mice** [58]
Virus	GII.4	GII.4, GII.12	GII.3	GI.1, GII.2, GII.4, GII.17	GII.3	GI.1	GII.4
Inoculation (route and virus quantity)	Oral 1.62×10^7 genomes	Oral/intranasal 10^4-10^{10} genomes	Intragastric 10^7 genomes	Oral/intragastric 10^5-10^6 genomes	Nasogastric, Quantity not clear	Intravenous/ intra-gastric 4×10^6-4×10^8 genomes	Intraperitoneal 4×10^3-7×10^4 genomes
Shedding	3 days	2-16 days	7 days	1-19 days	Up to 21 days	2 days-17 weeks	No shedding[1]
Seroconversion	Yes	Yes	NA	Yes/no[2]	Yes	Yes	No
Pathology	Lesions, mild villous atrophy, enterocyte vacuolization in small intestine	Increase in inflammatory cells in LM, necrosis, shortening of villous tips	No damage	No damage	NA	No damage	No damage
Tropism (detection of viral antigen or genome)	Positive enterocytes in the ileum, cells in LM	Enterocytes and cells in LM of duodenum, jejunum, and ileum. Spleen and MLN	Immune cells in the small/ large intestine, tonsils, spleen, lymph nodes, MLN	NA	NA	Cells in LM of duodenum and jejunum	Macrophage-like cells in, liver and spleen. Viral genomes detected in various tissue[3]
Disease	Diarrhea	Diarrhea	Diarrhea	Asymptomatic	Diarrhea	Asymptomatic	Asymptomatic
Viremia	Yes (low)	Yes	Yes	NA	NA	NA	NA

[1] When inoculated orally and intraperitoneal simultaneously, virus was shedded in feces; [2] Depending on study; [3] Stomach, small/large intestine, MSN, liver, spleen, kidney, heart lung, bone marrow. MSN=mesenteric lymph nodes, LM=lamina propria.

The best understood host factors influencing susceptibility to human norovirus infections are the HBGAs, glycans that act as attachment factors for norovirus, and the host secretor status [72, 271-274]. Alternative attachment factors, including sialic acids and heparan sulfate, have been proposed and it is likely that other cell surface molecules play a role in norovirus binding to the cell [275-278]. Virus attachment is a prerequisite for a cell's susceptibility to infection and studying a host's or population's HBGA distribution can imply putative target cells and susceptible populations, respectively; HBGA expression and distribution within a host can indicate virus cell tropism while their expression in different putative human and animal hosts can be an indicator for host range.

A host's HBGAs type is determined by the ABO- and Lewis blood group systems. ABO synthesis begins with the addition of fucose to a carbohydrate precursor on glycoprotein or to glycolipid precursor structures by a α1,2-fucosyltransferase. This enzyme is expressed from two separate loci (*H* and *Se*) one expressed on red blood cell precursors, the other on epithelia cells of the gastrointestinal, respiratory, and reproductive tract. Individuals who have a non-functional fucosyltransferase 2 (*FUT2*) version express the H antigen only on their red blood cells but not in their gastrointestinal tract. The A and B antigen are subsequently added onto the H antigen by various other glycosyltransferases. Lewis antigens are sugar moieties, consisting of a precursor structure, or the A, B, H antigens, to which an extra fucose group has been added. The *Se* locus also determines whether soluble forms of the ABH antigens are secreted into bodily fluids. Humans with an inactive *Se* gene are referred to as non-secretors since no ABH antigens are found in their saliva and mucus [279]. Noroviruses bind these HBGA in a strain dependent manner, thus leaving non-secretors non-susceptible to some norovirus strains. In pigs and primates, the HBGA phenotype seems to be important for infection with human norovirus as well. In animal studies the host's HBGA phenotype and virus strain used for inoculation can be selectively paired. Binding assays have been used as an alternative surrogate to study interaction between virus attachment factors (**Fig. 4** and **Table S3**).

Animal or human norovirus VLPs or purified virus can be tested with regards to their attachment to either animal or human saliva or tissue with known HBGA content. Canine and the newly discovered norovirus from bats appear to attach to HBGAs similar to human noroviruses [170, 248]. Bovine GIII.2 and murine GV have been shown to be dependent on receptors that are not thought to be expressed in humans; GIII.2 strains do not bind the same sugar moieties as human norovirus but to αGal 1-3 sugar (Galα3Galβ4GlcNAcβ-R) instead [280]. This epitope is expressed in all mammals with the exception of the *Hominidae* family. In line with this, GIII.2 particles bound strongly to bovine saliva but neither to human saliva nor duodenal tissue.

GV infection in mice was reported to depend on terminal sialic acids and glycoproteins on macrophages, in a strain dependent manner [281]. Recently, a proteinaceous receptor, CD300lf, was detected in mice, which is expressed on tuft cells that are present in small numbers in the intestine as well as on cells of the hematopoietic/myeloid lineage. However, the human CD300lf homologue does not function as receptor for human or murine norovirus [282, 283]. For other noroviruses, including porcine genotypes and feline genotypes, no attachment factor or receptor is known.

Most of the susceptible hosts mentioned above, with the exception of several fish and bird species, contain a *FUT1* and *FUT2* gene. The lack of these genes can be potentially compensated for by another fucosyltransferases, or alternatively in these newly discovered animals norovirus, could attach to an alternative receptor [284].

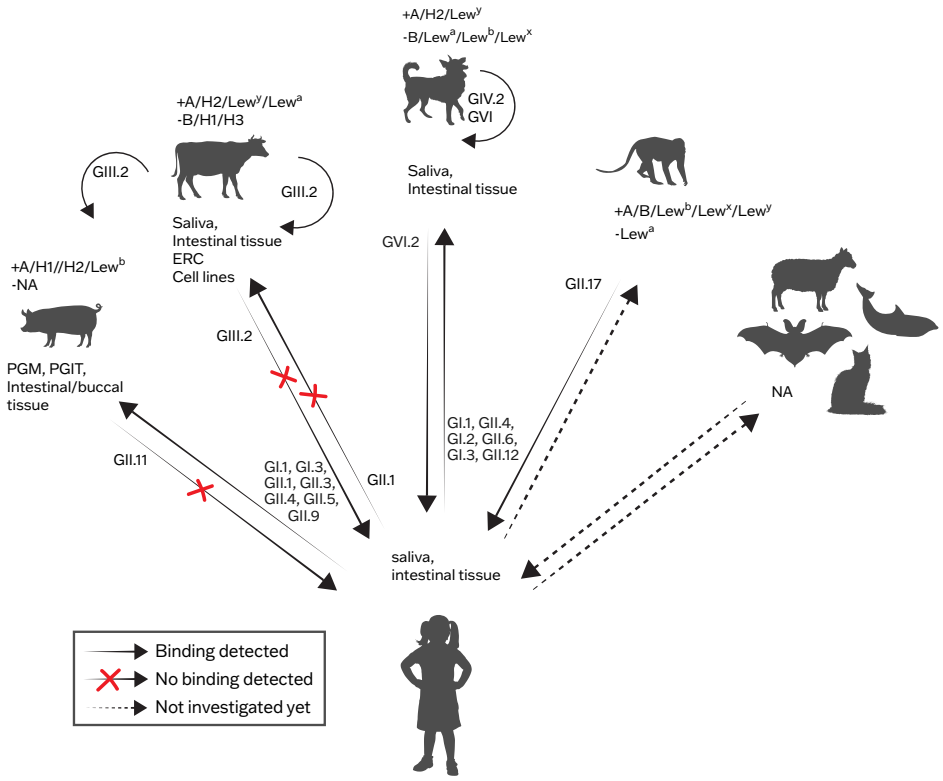

Figure 4. Results of binding studies with animal and human norovirus VLPs. The histo-blood group antigen (HBGA) phenotype is indicated with the presence (**+**) or absence (**-**) for different glycans. Arrows indicate direction in which attachment was tested and whether attachment was observed or not (**red cross**). Dotted arrows indicate that attachment has not been assessed yet. The half circular arrows indicate binding of animal norovirus to tissue/saliva of either the same or another animal species. Detailed information about the individual studies can be found in **Table S3**.

3. Discussion and Conclusions

More than two thirds of emerging infectious diseases that affect humans originate from animal reservoirs, predominantly bats, rodents, birds, and other wildlife, and therefore, we sought to review evidence for interspecies transmission of noroviruses [285]. While most of our understanding about the norovirus animal reservoir stems from domestic animals, the recently increasing number of metagenomic studies, investigating the virome in a more unbiased way, have extended the norovirus host range by new species, while simultaneously complementing the knowledge about norovirus diversity. For many of these newly discovered viruses, we have little more information than a genome sequence and it remains to be determined if they indeed are host specific. Bats, wild rodents, and birds are known to frequently host pathogens that can cause disease, but have hardly been studied for evidence of norovirus infection.

Our review found more evidence for human noroviruses in animals than the reverse, suggesting that human norovirus could be a reverse zoonosis, with identification of human norovirus RNA in stool samples from pets, rodents, birds, pigs, and cattle. However, the question is what constitutes evidence for infection, as it can be argued that the detection of norovirus in feces indicates ingestion of norovirus contaminated material rather than an active infection. The molecular RNA detection methods can be sensitive enough to detect amounts as low as 10 virus genomes and such low virus levels could be due to ingestion [286]. To establish that both species can serve as a host, detection of either replicating virus by increase in virus titer over time, a specific immune response, or detection of proteins that are only expressed upon infection is required. This has been shown experimentally in cattle, pigs, macaques, and chimpanzees, confirmed by seroconversion and virus shedding. Serological studies can also be used to confirm viral detection in field studies, thus increasing the window of detection, as antibodies persist much longer than virus shedding. However, serological assays have their draw backs: antibodies can potentially also be induced by exposure to the virus rather than infection and cross-reactivity has to be taken into consideration when analyzing the results. Cross-reactivity has been described primarily between strains within one genogroup and less between viruses from separate genogroups [158]. This is of importance when analyzing serology data against human and animal noroviruses that cluster in the same genogroups, such as porcine, feline, and canine noroviruses. Many serology studies reported some sera that contained antibodies only recognizing animal strains but not humans or *vice versa*, increasing the chance that these are specific antibody responses. Serology has the advantage of providing information about the prevalence of a pathogen in a certain host species without relying on samples to be taken during an active infection. It is therefore a good tool to screen potential hosts with regards to their risk of exposure. However, this data should be complemented by detection of viable virus from the host. Since culturing

is difficult for norovirus, deep sequencing to detect viral genomes is for now the best alternative. Should human norovirus infect animals the question remains whether these interspecies transmissions are relevant for human infections; if once transmitted to animals, these strains can be re-introduced into humans. Furthermore, strains that only cause sporadic infection in humans, such as GIV noroviruses, could reside in an animal reservoir between outbreaks.

Evidence for transmission of animal norovirus to humans is sparse and solely based on serological evidence. If these transmissions occur, they are likely to be rare events that could be difficult to detect if they are asymptomatic or sporadic infections. In addition, surveillance is not developed to detect these viruses in human stool samples. Several papers reported differences in detection rate based on their choice of primers; protocols with GI or GII specific probes will potentially miss the animal noroviruses, while the generic calici- or norovirus primers that are often used for detection of human and animal noroviruses in animals might have lower sensitivity compared to more specific primers [199, 237, 240, 263, 287, 288]. It is open to debate whether some viruses that are categorized as human norovirus today might have originated from an animal source; the origin of newly emerging recombinants, such as the GII.pe polymerase, is unknown and it is a possible scenario that these new recombinants are the result of a recombination event between an animal and a human norovirus. Recombination occurs primarily within genogroups and only three intergenogroup recombinants namely between GI.3-GII.4, GII(NA)-GVI, and feline GIV.2-GVI.1, have been identified [245, 289, 290]. Recombinants are also found within bovine, porcine, canine, and feline genotypes. The formation of human-animal norovirus recombinants is a possible scenario, especially for animal genotypes that cluster close together with human genotypes. Water, food sources, and filter feeding shellfish can harbor a variety of multiple human and animal genotypes and genogroups simultaneously thereby posing a possible source of co-infection in humans and animals [291-294]. To increase chances of catching a transspecies transmission event more targeted surveillance would be needed; to include samples of animals and humans that are in close contact, ideally during an outbreak situation and with an unbiased detection method [150, 251, 252, 295]. Serology has the advantage of providing a broader view of exposure to certain pathogens without relying on samples to be taken during an active infection. It is therefore a good method to screen broadly in potential hosts and identify certain populations with regards to their risk of exposure; e.g., animals that are in close contact to humans. To confirm the infection however the virus should ideally be cultured to show its viability. This is still difficult for norovirus and to identify at least viral genomes by deep sequencing is therefore used as to confirm the presence of norovirus in the host. Unravelling norovirus reservoirs and movement between species will help us understand norovirus evolution and emergence.

4. Materials and Methods

4.1. Search strategy

We searched the literature in the Embase, Medline Ovid, Web of science, and Google scholar databases, using the search strings shown below. Number of papers found is depicted in brackets.

4.1.1. embase.com (2903)

("Norovirus"/exp OR "norovirus infection"/exp OR (Norovirus* OR Norwalk OR "small round-structur*" OR srsv*):ab,ti) AND ([animals]/lim OR "reservoir"/exp OR (nonhuman/de NOT human/exp) OR "zoonosis"/de OR "disease model"/de OR (animal* OR reservoir* OR nonhuman*OR non-human* OR animal* OR rat OR rats OR mouse OR mice OR murine OR dog OR dogs OR canine OR cat OR cats OR feline OR rabbit OR cow OR cows OR bovine OR rodent* OR sheep OR ovine OR pig OR swine OR porcine OR veterinar* OR chick* OR baboon* OR nonhuman* OR primate* OR cattle* OR goose OR geese OR duck OR macaque* OR avian* OR bird* OR mammal* OR poultry OR bat OR porpoise* OR zoono* OR farm OR farms OR "disease model*"):ab,ti)

4.1.2. Medline Ovid (1550)

(Norovirus/OR (Norovirus* OR Norwalk OR small round-structur* OR srsv*).ab,ti.) AND ((exp animals/NOT exp humans/) OR Disease Reservoirs/OR Zoonoses/OR Models, Animal/OR Disease Models, Animal/OR (animal* OR reservoir* OR nonhuman* OR non-human* OR animal* OR rat OR rats OR mouse OR mice OR murine OR dog OR dogs OR canine OR cat OR cats OR feline OR rabbit OR cow OR cows OR bovine OR rodent* OR sheep OR ovine OR pig OR swine OR porcine OR veterinar* OR chick* OR baboon* OR nonhuman* OR primate* OR cattle* OR goose OR geese OR duck OR macaque* OR avian* OR bird* OR mammal* OR poultry OR bat OR porpoise* OR zoono* OR farm OR farms OR disease model*).ab,ti.)

4.1.3. Web of Science (2049)

TS = (((Norovirus* OR Norwalk OR "small round-structur*" OR srsv*)) AND ((animal* OR reservoir* OR nonhuman* OR non-human* OR animal* OR rat OR rats OR mouse OR mice OR murine OR dog OR dogs OR canine OR cat OR cats OR feline OR rabbit OR cow OR cows OR bovine OR rodent* OR sheep OR ovine OR pig OR swine OR porcine OR veterinar* OR chick* OR baboon* OR nonhuman* OR primate* OR cattle* OR goose OR geese OR duck OR macaque* OR avian* OR bird* OR mammal* OR poultry OR bat OR porpoise* OR zoono* OR farm OR farms OR "disease model*")))

4.1.4. Google Scholar (200)

Norovirus | Norovirusses | Norwalk | "smallround-structur" | srsv animal | animals | reservoir | nonhuman | zoonosis | zoonoses | "disease model"

4.2. Selection criteria

Two independent reviewers screened titles and abstracts for their relevance. We included publications that mentioned norovirus in the title or abstract but we excluded papers about food (oyster) and waterborne outbreaks, food surveillance or food related experiments, and oyster/seafood surveillance. We excluded papers on murine noroviruses as models. Papers describing norovirus surveillance in wild mice and papers using mice as model for human norovirus were included (**Fig. 5**). In a second round, we screened the papers for whether they described (1) animal surveillance studies to detect human or animal norovirus by PCR, sequencing or by serosurveillance including negative results; (2) experimental animal infections with

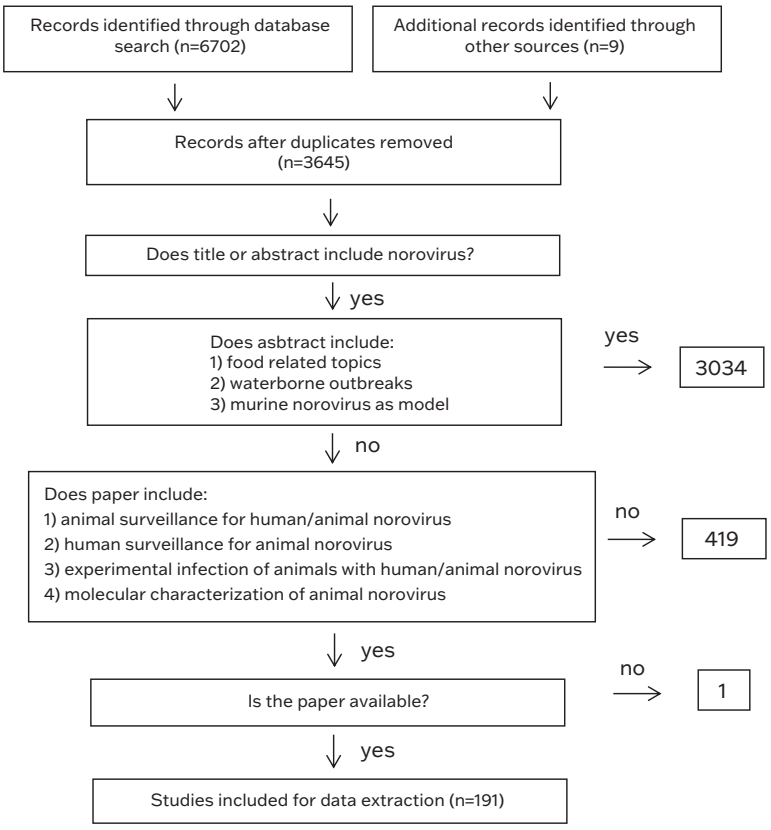

Figure 5. Inclusion and exclusion criteria for paper selection.

human or animal norovirus; (3) human surveillance studies to detect animal norovirus by PCR, sequencing or by serosurveillance including negative results; (4) animal norovirus characterization including molecular assays and genome announcements.

4.3. Data extraction

Of the remaining papers, the following data was extracted:

1. General description. Location (country, district, city), duration of study, date of study, species and number of tested animals and age of animals. For studies describing experimental infections of animals with human or animal noroviruses, the following information was collected if described in the paper:

2. Details on experimental infection methods. Regarding the experimental infection, the route of inoculation was documented since this may affect which subclasses of immunoglobulins are induced. In addition, genogroup/genotype of the virus inoculate, as well as amount used (number of genome copies) and the sample type collected (e.g., saliva, feces, sera) were registered. It was further recorded how virus replication was confirmed, which methods was used to detect virus (RT-PCR, real-time RT-PCR, antigen capturing ELISA, EM), how much was detected and at what time points.

3. Details on clinical picture; description of the health state of the animals; which symptoms (e.g., diarrhea, vomiting), as well as the duration of symptoms.

4. Pathology; pathological examination results.

5. Immunohistochemistry data was extracted to for information regarding the organ and cell tropism.

6. Host response was assessed by collecting serological data including method of antibody detection, type of immunoglobulins (Igs) tested (IgM, IgG, IgA), origin of Igs (saliva, sera, feces), the time period Igs were detected and if available whether they were blocking virus from binding to HBGAs. Since some animal noroviruses cluster close to human norovirus, information about cross-reactivity was also collected. Host factors such as HBGA, secretor and non-secretor status were of interest, since they are known to be important for susceptibility in humans, while in animals this link is less evident.

For surveillance studies additional data was collected regarding duration of surveillance, species, setting of the animals (farm, slaughterhouse, research facility, households, and the wild), and type of farm (if applicable; indoor/outdoor/free range). When virus shedding was detected by RT-PCR, it was noted which region of the genome was detected and whether the ORF1/ORF2 overlap was amplified. Furthermore, the similarity of new virus sequences with known sequences in the database was recorded. When sequences were available, they were re-typed with the NoroNet typing tool.

Supplementary Materials: http://www.mdpi.com/1999-4915/11/5/478/s1.

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Author Contributions: Conceptualization, N.V., M.P.G.K. and M.d.G.; methodology, N.V., M.P.G.K. and M.d.G.; investigation, N.V. and M.d.G.; writing, original draft preparation, N.V. and M.d.G.; writing, review and editing, N.V., M.P.G.K. and M.d.G.; visualization, N.V. and M.d.G., supervision, M.P.G.K. and M.d.G.; project administration, N.V., M.P.G.K. and M.d.G.; funding acquisition, M.P.G.K.

Conflicts of Interest: The authors declare no conflict of interest.

Supplementary material

Table S1. Studies investigating animal norovirus in animals and the detection methods that were applied. Included are studies that analyzed fecal samples by RT-PCR, real-time RT-PCR or EM and serological studies. Frequently used primers sets are p289/p290 and p290/p110 (universal calicivirus primers, RdRp), JV12/JV13 (norovirus specific), PNV7/PNV8 (porcine norovirus specific, RdRp), G2SKF/G2SKR (GII, capsid), MON432/MON477 (norovirus), FNoV-F9 and FNoVR15 (GIV specific), JV102/JV103 (canine norovirus specific), CBeCu-F/R (bovine norovirus specific). Unless otherwise stated sample were collected at one time point. MNV = murine norovirus, EM = electron microscopy.
Sample type: F=Feces, FP=Pooled feces, S=Sera, IC=Intestinal content [a] longitudinal study; [b] case control; [c] outbreak.
Age, Clinical Status: A = adult (in pigs >6month), J = juvenile, +=symptomatic, -=asymptomatic

Location	Host	Investigated genogroup, genotype	Animal norovirus detected	Prevalence (%)	Detection method	Primers, probes and antigens used	Samples Type	Samples Location (n)	Age Clinical status	Ref
The Americas										
USA	Pigs	GII.11/18/19	GII.11/18/19	64/621 (10)[1]	RT-PCR, microwell hybridization	PNV7/PNV8, Hybridization probes specific for GII.11/18/19	F[a]	Farms #7 Abattoirs #1	J/A-	[338]
	Pigs	GII.11	GII.11	78/110 (71)	ELISA	VLPs of GII.11 (SW918)	S	Farms Abattoirs #1	NA	[211]
		calicivirus	none	0/104 (0)	RT-PCR	p289H,I-p290H,I,J,K	F/IC	Farms #1 Abattoirs #2	J-	
	Pigs	GII.11/18/19	GII.11/18	78/413 (19)	RT-PCR, microwell hybridization	PNV7/PNV8, G2SKF/G2SKR. Hybridization probes specific for GII.11/18/19	PF	Farms #9	A-	[193]
	Pigs	calicivirus	GII.11/18/19	6/275 (2)	RT-PCR	p290/p110	F	Farms #6 Abattoirs #1	A-	[190]
	Pigs	calicivirus	none	0/139 (0)	RT-PCR	p290/p110, G2SKFM-Po/G2SKRM-Po, JV12Y/JV13I	F	Farms #3	J	[296]
	Cattle	GIII.2	GIII.2	231/231 (100)	ELISA	VLPs of GIII.2 (AF542084)	S[a]	Feedlots #3	J	[297]*
	Cattle	calicivirus	GIII.1/2	54/75 (72)	RT-PCR	NV35/36, p290/p289 JV12/13 J11 U/L, J21 U/L, BEC-POL5/3, NBU-F/R, CBECU-F/R,	RS[a,b]	Farms #2	J	[298]*
	Cattle	GIII	GIII	128/444 (29)	real-time RT-PCR	BNoV-fwd/rev (1014)	F[b]	Farms #140	J+/-	[219]

Location	Host	Investigated genogroup, genotype	Animal norovirus detected	Prevalence (%)	Detection method	Primers, probes and antigens used	Samples Type	Samples Location (n)	Age Clinical status	Ref
USA	Cattle	norovirus	GIII.1/2	52/74 (70)	RT-PCR	MON432/MON477, CDC primer set	F	Farms #22	J+	[220]
	cats	calicivirus	GIV.2	6/24 (43)	RT-PCR	p289/p290, FNoV-F9/FNoVR15	F[c]	Clinic #1 Shelter #1	J/A+/-	[240]
	cats	all	GIV	1/6 (17)	RT-PCR	metagenomics, random primers	PF	Shelter #1	NA	[244]
	Sea lion	all	GII/GIV	4/47 (9)	RT-PCR	metagenomics, random primers	F	Marine mammal centre #1	J/A	[247]
Canada	Pigs	norovirus	GII.11/18	26/120 (22)	RT-PCR	Region B and A primer MON431-MON434 (GI, GII), G1/G2 (SRSV)	PF[a]	Farms #10	NA	[195]
	Cattle	norovirus	GIII.2	2/179 (1)			PF	Farms #45	J/A	
	Pigs	calicivirus	GII.11/18	17/20 (85)[2]	RT-PCR	p289/p290, p289N/p290N	PF/F	Farms #20	J/A	[196]
	Pigs			12/66 (18)			IC[3]	Abbattoirs #2	A-	
	Pigs	calicivirus	porcine NoV	4/200 (2)	real-time RT-PCR, RT-PCR	p289/p290, qRT-PCR COG2F/COG2R (GII), probe RING2	F	Farms, Abattoirs	N	[197]
Venezuela	Pigs	calicivirus	none	0/137 (0)	RT-PCR	p289/p290, GLPSG1/YGDDI, GLPSG2/YGDD1	F	Farms #7	J +/-	[204]
	Cattle	GIII	GIII	1/129 (1)	RT-PCR	NA	F	NA	NA	[299][12]
Argentina	Cattle	GIII	GIII.1/2, Not typed	3/90 (3)	RT-PCR	CBECu-F/CBECu-R	F	Farms	J+	[300]
Brazil	Pigs	GII.11/18/19	GII.11	58/112 (52)	RT-PCR	SwNV1/SwNV2	F	Farms	A-	[209]
	Pigs	calicivirus	GII.18	1/96 (1)	RT-PCR	p289/p289HI, p290/290HIJK, Mon431/432, 433/434	RS	Farms #5	J/A +/-	[301]
	Pigs	calicivirus	GII.11/18/19	20/261 (7)	RT-PCR	p289/p289HI, p290/p290HIJK, Cap C/D3/D1	F/IC	Farms #6, Abattoirs #1	J/A +/-	[302]
	Pigs	calicivirus	none	0/169 (0)	RT-PCR	p289/p290	RS	Farms #5#	J+/-	[303]
	Pigs	calicivirus	GII.11	2/30 (7)	RT-PCR	p289/p290, SwNV1/SwNV2	RS[c]	Farms #4#	J+	[304]

Location	Host	Investigated genogroup, genotype	Animal norovirus detected	Prevalence (%)	Detection method	Primers, probes and antigens used	Samples Type	Samples Location (n)	Age Clinical status	Ref
Brazil	Cats	calicivirus	GIV.2	1/29 (3)	RT-PCR, Real-time RT-PCR	p289H/p290H, FNoV-09/FNoV-R15, Mon4FR, Probe Ring4	F[c]	Shelters #4	J+	[243]
Asia/New Zealand/Australia										
China	Pigs	calicivirus	none	0/209 (0)	RT-PCR	p289/p290	F	Farms #6	J/A-	[198]
	Pigs	GII.11/18/19	GII.11 - NA	2/12 (17)	RT-PCR	not stated	RS	Farms #3	J+	[191, 192]
	Pigs	GII.11	GII.11	2/904 (0,2)	RT-PCR	p289/p290	F	Farms #14	J/A	[200]
	Dogs	NA	GVII	2/NA (NA)	NA	NA	RS	NA	NA	[339]
	Cattle	Bovine noro-virus	GIII.1	3/28 (11)	RT-PCR	CBECU-F/CBECU-R	F	Farms #5	J+	[229]
	Bats	calicivirus	Not typed	NA	metagenomics	NA	RS	Outdoors	NA	[249]
	Bats	Not typed	Not typed	2/62 (3)	RT-PCR	Primers designed based on newly found virus	RS	Outdoors	A-	[250]
	Bats	norovirus	NA	10/235 (4)**	metagenomics	Primers designed based on newly found virus	IC	Outdoors	A-	[175]
	Brown rat	NA	GV	NA	RT-PCR	NA	RS	NA	NA	[340]
Taiwan	Pigs	calicivirus	GII.11	9/533 (2)	RT-PCR	p290/p110, NV2oF2/NV2oR, G2F3/G2SKR	F	Farms #6	J/A-	[199]
Japan	Pigs	GII.11		95/266 (36)	ELISA	VLPs of GII.11 (SW918)	S	NA	J	[211]
	Pigs	calicivirus	GII.11/18/19	36/240 (15)	RT-PCR, real-time RT-PCR	p289/p290, G1SKF/G1SKR, G2SKF/G2SKR qRT-PCR: COG2F/ALPF/COG2R	IC[a]	Farms #3 Abattoirs #1	A-	[201]
	Pigs	norovirus	GII.11	4/1117 (0.4)	RT-PCR	35/36 (SRSV), NV81/NV82	IC[3]	Farms #26	-	[179]
	Pigs	calicivirus	GII.11	1/24 (4)	RT-PCR	p289/p290	IC	Diagnostic lab #1	J+/-	[210]

Location	Host	Investigated genogroup, genotype	Animal norovirus detected	Prevalence (%)	Detection method	Primers, probes and antigens used	Samples Type	Samples Location (n)	Age Clinical status	Ref
Japan	Dogs	calicivirus	GIV (NA)	2/97 (2)	RT-PCR	p290d/p289d	F	Households	+	[242]
	Cats		GIV.2	1/83 (1)						
	Cats	caliciviurs	GIV.2	1/NA (NA)	RT-PCR	p290d/p289d	RS	Shelter	-	[245]
	Mice [11]	GV	GV	6/44 (14)	RT-PCR	MNV-F1/MNV-R1, MNV-F2/MNV-R2	IC	Outdoors	NA	[305]
	Rat[12]		GV	1/1 (100)						
	Grass vole[5]		none	0/1 (0)						
Korea	Pigs	norovirus	GII.11/18	10/537 (2)	RT-PCR	GIIF2/GIIR, GIIF1/GIIF2, GIIF2/GIIR	F	Farms #64	J/A +/-	[306]
	Pigs	norovirus	GII.11/18	3/567 (0,5)	RT-PCR	NORO-DG35OF/IF	F	Farms #12	NA	[202]
	Dogs	Canine norovirus	GIV.2	14/459 (3)	RT-PCR	JV102/JV103 ELISA: p-domain of GVI.2 (GQ443611)	F/S	Clinic, Shelters	+/-	[307]
			GVI.2	68/427 (16)	ELISA					
	Cattle	GIII	GIII.1/2	60/645 (9)	RT-PCR	bovine norovirus-specific primers	F	Farms #629	J+	[230]
Iran	Cattle	GIII	GIII.1/2	100/253 (40)	RT-PCR	CBECU-F/CBECU-R	F[a]	Farms #42	J+	[308]
	Cattle	GI, GII	GIII	9/50 (18)	RT-PCR	Ni/E3	F	Farms #6	+	[231]
Turkey	Cattle	GIII	GIII.2	6/70 (9)	real-time RT-PCR	IIIBoVo-F/R	F	Farms	J+	[232]
	Cattle	GIII	GIII.2	5/127 (4)	RT-PCR	BoNoV851-F/BoNoV1350-R	RS	NA	J+	[309]
India	Cattle	GIII	GIII.1	1/249 (0,4)	RT-PCR	BEC-POL NBU-F/R CBECU-F/R, J11U/CBECU-R	F	NA	+	[147]
New Zealand	Pigs	GI-GIII	GII.11	2/23 (9)	RT-PCR	primers that detects GI-GIII	F	Farms #2	J-	[203]
	Cattle	GI-GIII	GIII.1	15/28 (54)	real-time RT-PCR	newly designed based on 52 NoV sequences	F	Farms #2	-	[310]
	Sheep	GI-GIII	GIII.3	8/33 (24)	RT-PCR	primers that detects GI-GIII	F	Farms #2	J-	[203]

Location	Host	Investigated genogroup, genotype	Animal norovirus detected	Prevalence (%)	Detection method	Primers, probes and antigens used	Samples Type	Samples Location (n)	Age Clinical status	Ref
Australia	Cattle	calicivirus	GIII.2	2/8 (25)	RT-PCR	p289/p290, CBECU-F/ CBECU-R	PF	Farms	J+	[341]
Europe										
Italy	Pigs	GII.11/18/19	none	0/242 (0)	RT-PCR	PNV7/PNV8	F	Farms #8	J/A +/-	[311]
	Pigs	calicivirus	GII.11	1/201 (0,5)	RT-PCR	p290/p289/p110, PNV7/ PNV8	F	Farms #15	J/A -	[288]
			none	0/89 (0)			F	Farms #2	J+	
	Cattle	GIII	GIII.1/2	11/104 (11)	RT-PCR	CBECU-F/R		Farms #16	J-	[221]
	Cattle	GIII	GIII.1/2	21/101 (21)	RT-PCR	JV12/13 J11 U/L, J21 U/L, BEC-POL NBU-F/R, CBECU-F/R, J11U/CBE-CU-R	F	Farms	J-	[222]
	Dogs	GIV.2, GVI.2	total	66/516 (5)	ELISA	VLPS of GIV.2 (EF450827), GVI.2 (JF930689)	S	Households	J/A	[254]
			GIV.2	20/516 (4)						
			GVI.2	46/516 (9)						
	Dogs	calicivirus	GIV.2	4/183 (2)	RT-PCR	p289/p290, JV12Y/JV13I		NA	J+	[312]
	Dogs	GIV.2	GIV.2	5/103 (5)	ELISA	VLP of GIV.2 (EF450827)	S	NA	A	[239]
Italy	Dogs	GVI.2	GVI.2	6/10 (60)	ELISA	VLPs of GVI.2 (GQ443611)	S	Clinics	J/A	[238]
	Dogs	calicivirus	GVI.2	11/239 (5)	RT-PCR	p298/p290, JV12Y/JV13I	F	Households	J+/-	[236]
	Dogs	calicivirus	GIV.2	1/1 (100)	RT-PCR	p289/p290	F	NA	J+	[342]
	Lion	calicivirus	GIV.2	1/1 (100)	real-time RT-PCR, RT-PCR	p290/p289, JV12Y/JV13I	F	Zoo #1	J+	[233]
	Cats	calicivirus	GIV.2	3/105 (3)	RT-PCR	p289/p290, JV12Y/JV13I	F	Shelters #3	J+/-	[241]
	Cats	GIV.2	GIV.2	34/211 (16)	ELISA	VLP of GIV.2 (EF450827)	F	Clinics, Shelter, outdoors	A	[239]
Spain	Pigs	calicivirus	NA	27/221 (12)	real-time RT-PCR, RT-PCR	p289/p290, JV12/JV13. qRT PCR: probes for GI and GII	F	Farms #14 Abattoirs #1	J/A+/-	[313]
	Dogs	calicivirus	GIV.2	2/26 (8)	RT-PCR	p298/p290, JV12Y/JV13I	F	Households	J+/-	[236]

Location	Host	Investigated genogroup, genotype	Animal norovirus detected	Prevalence (%)	Detection method	Primers, probes and antigens used	Samples Type	Samples Location (n)	Age Clinical status	Ref
Portugal	Dogs	Canine Norovirus	GIV.2	7/7 (100)	RT-PCR	JV102/JV103	F[c]	Kennel #1	J+	[234]
	Dogs	calicivirus	GVI.2	29/105 (28)	RT-PCR	p289/p290, JV12Y/13I JV102/JV103	F	Shelters, shops, clinics	+/-	[237]
	Dogs	Canine noro-virus	GIV.2	60/256 (23)	RT-PCR	JV102/JV103	F	shelter, shops clinic	+/-	[235]
	Dogs	GVI.2	GVI.2	64/100 (64)	ELISA	VLPs of GVI.2 (GQ443611)	S	Clinics	J/A	[238]
Greece	Dogs	calicivirus	GIV.2	6/72 (8)	RT-PCR	p289/p290, JV12Y/JV13I	F[c]	Kennels, pet shop, clinic	J+	[314]
France	Dogs	GVI.2	GVI.2	10/50 (20)	ELISA	VLPs of GVI.2 (GQ443611)	S	Clinics	J/A	[238]
	Dogs	calicivirus	none	0/26 (0)	RT-PCR	p298/p290, JV12Y/JV13I	F	Households	J+/-	[236]
	Cattle	GIII	GIII.1/2	89/456 (20)	RT-PCR	CBECU-F/CBECU-R	F[c]	Farms #415	J+	[225]
	Cattle	GIII	GIII.1/2	29/81 (36)	RT-PCR	CBECU-F/CBECU-R	F[b]	Farms NA	J+	[226]
Switzerland	Dogs	GVI.2	GVI.2	2/10 (20)	ELISA	VLPs of GVI.2 (GQ443611)	S	Clinics	J/A	[238]
Germany	Pigs	GI, GII	GII.18	17/120 (14)	RT-PCR	Mon431-434	F	Abattoirs #3	A	[206]
	Cattle	GIII	GIII.1	66/100 (66)	ELISA	VLPs of GIII.1 (AJ011099), GIII.2 (AF320625)	S	Farms #12	A	[315]
			GIII.2	87/100 (87)						
			GIII.1	71/100 (71)			S	Farms #6	J	
			GIII.2	94/100 (94)						
	Cattle	norovirus	GIII.2	409/439 (93)	RT-PCR	NV32/NV36, NV33/ NV35, JV12/JV13 BECF,-BECR	F	Farms #29	J/A+	[317]
	Cattle	GIII.1	GIII.1	817/824 (99)	ELISA	VLPs of GIII.1 (AJ011099)	S	Farms #25	J/A	[316]
				34/381 (9)	antigen ELISA,	Anti-GIII.1 VLP hyperim-une sera	F	Farms	+	

Location	Host	Investigated genogroup, genotype	Animal norovirus detected	Prevalence (%)	Detection method	Primers, probes and antigens used	Samples Type	Samples Location (n)	Age Clinical status	Ref
Germany	Dogs	GVI.2	GVI.2	8/50 (16)	ELISA	VLPs of GVI.2 (GQ443611)	S	Clinics	J/A	[238]
	Dogs	all	GIV.2	13/294 (4)	RT-PCR	p298/p290, JV12Y/JV13I	F	Households	J+/-	[236]
	Rat[10]	all	GV	2/20 (10)	NGS		IC	Outdoors	NA	[318]
Netherlands	Pigs	norovirus	GII.11	2/100 (2)	RT-PCR, Southern blot	JV12/JV13	PF	Farms #100	A	[178]
	Cattle		none	0/43 (0)			PF	Farms #75	J	
	Cattle		GIII.2	25/75 (44)			PF	Farms #43	A	
	Chicken		none	0/48 (0)			PF	Broilers #48	J	
	Cattle	norovirus	GIII.2	77/243 (32)	RT-PCR, EM, Southern blot,	JV12/JV13	PF	Farms	J	[319]
	Cattle	norovirus	GIII.2	13/312 (4)			F[a]	Farms #1	J+/-	
	Dogs	GVI.2	GVI.2	17/50 (34)	ELISA	VLPs of GVI.2 (GQ443611)	S	Clinics	J/A	[238]
	Porpoise	HPNV	NA	5/49 (10)	Real-time RT-PCR	HPNV primers, GI probe	IC[7]	NA	NA	[62]
Belgium	Pigs	calicivirus	GII.19	2/43 (5)	RT-PCR	JV12/JV13, p289/p290, swNo F/R	F	Diagnostic #1	J/A+/-	[287]
	Cattle	calicivirus	GIII.2	10/133 (8)	RT-PCR	p298/p290, CBECu , BEC, JV12/JV13	F[a]	Diagnostic #1	J+	[223]
		GIII.2	GIII.2	409/439 (93)	ELISA	VLPs of GIII.2 (EU794907)	S[b]		J/A	
	Cattle	norovirus	GIII.2	15/317 (5)	RT-PCR	JV12/JV13 (all NV), CCV3/CCV4, CBECU-F/ CBECU-R	F	Farms	NA	[343]
	Cattle	GIII	GIII.1, GIII.2	28/300 (9)	RT-PCR	CBeCu-F/R (bovine NoV), AMG1-F/R (based on GIII.1) sequence	F	Diagnostic #1	J+	[224]
	Cattle	GIII	GIII.2	NA	RT-PCR	CBeCu-F/R	F	Diagnostic	J	[320]

Location	Host	Investigated genogroup, genotype	Animal norovirus detected	Prevalence (%)	Detection method	Primers, probes and antigens used	Samples Type	Samples Location (n)	Age Clinical status	Ref
UK	Pigs	calicivirus	GII.19	2/43 (5)	RT-PCR	JV12/JV13, p289/p290, swNo F/R	F	Diagnostic #1	J/A+/-	[287]
			GIII.2	87/100 (87)						
	Cattle		GIII.1	98/100 (98)			S	Farms #7	J	
			GIII.2	66/100 (66)						
	Cattle	NA	GIII	44/398 (11)	Meta analysis	NA	F	Diagnostic	NA	[321]
	Dogs	calicivirus	none	0/227 (0)	Real-time RT-PCR	Primers and probes based on GIV.2, GVI.1, GVI.2	F[b]	Clinics #4, Kennels	J/A+/-	[322]
		GIV.2, GVI.1/2	total	189/396 (48)	ELISA	VLPs of GIV.2 (EU224456), GVI.2 (GQ443611), GVI.1 (FJ692501)	S[b]	Kennel #1 Research Institut #1	NA	
			GIV.2	47/396 (12)						
			GVI.1	78/396 (20)						
			GVI.2	8/396 (2)						
	Dogs	canine norovirus	none	0/248 (0)	Real-time RT-PCR	Canine specific primers and probes	F[b]	Clinics #6, Shelter #1	J/A+/-	[255]
		GIV.2, GVI.1, GVI.2	GIV.2, GVI.1, GVI.2	147/325 (45)	ELISA	VLPs of GIV.2 (EU224456), GVI.2 (GQ443611), GVI.1 (FJ692501)	S[b]	Kennel #1 veterinary college #1	NA	
	Dogs	GVI.2	GVI.2	23/50 (46)	ELISA	VLPs of GVI.2 (GQ443611)	S	Clinics	J/A	[238]
	Mice[4]	norovirus	GV	57/192 (67)	RT-PCR	GV specific, degenerate primers	F/RS[6]	Indoors	NA	[323]
	Mice[5]			11/51 (22)			F[6]	Outdoors	NA	
	Pigs	calicivirus	none	0/292 (0)	RT-PCR	p289/p290	F	Farms #4	J-	[205]
	Dogs	GVI.2	none	0/10 (0)	ELISA	VLPs of GVI.2 (GQ443611)	S	Clinics	J/A	[238]
Ireland	Pigs	calicivirus	none	0/292 (0)	RT-PCR	p289/p290	F	Farms #4	J-	[205]
	Dogs	GVI.2	none	0/10 (0)	ELISA	VLPs of GVI.2 (GQ443611)	S	Clinics	J/A	[238]

Location	Host	Investigated genogroup, genotype	Animal norovirus detected	Prevalence (%)	Detection method	Primers, probes and antigens used	Samples Type	Samples Location (n)	Age Clinical status	Ref
Denmark	Rat[10]	calicivirus	none	0/11 (0)	RT-PCR	p289CVa/p289CVb, p289CVc/p289CVd	IC	Sewer system #5	NA	[267]
	Dogs	GVI.2	GVI.2	10/50 (20)	ELISA	VLPs of GVI.2 (GQ443611)	S	Clinics	J/A	[238]
Sweden	Dogs	GVI.2	GVI.2	4/10 (40)	ELISA	VLPs of GVI.2 (GQ443611)	S	Clinics	J/A	[238]
Norway	Cattle	GIII	GIII.1, GIII.2	208/419 (50)	sybr green RT-PCR	BoNoV72F/BoNoV72R2 CBECU-F/BoNoV72R2	F	Farms #190	J+/-	[215]
	Dogs	GVI.2	GVI.2	16/50 (32)	ELISA	VLPs of GVI.2 (GQ443611)	S	Clinics	J/A	[238]
Finland	Dogs	GI, GII, GIV	none	0/92 (0)	real-time RT-PCR	COG2R/QNIF2 QNIFS for GII, in house primers and QNIF4/QNIF3 for GI, Mon4F/Mon4R and Ring4 for GIV	F	Household	J/A+/-	[253]
	Dogs	GVI.2	GVI.2	7/10 (70)	ELISA	VLPs of GVI.2 (GQ443611)	S	Clinics	J/A	[238]
Poland	Dogs	GVI.2	GVI.2	16/50 (32)	ELISA	VLPs of GVI.2 (GQ443611)	S	Clinics	J/A	[238]
Slovenia	Pigs	calicivirus	GII.11/18	5/406 (1)	RT-PCR	p290/NVp110, JV12Y/JV13I, JV12Y/NVp110 G1SKF/R, G2SKF/R	F	Farms #8	J/A-	[207]
	Cattle		GIII.2	2/108 (2)			F	Farms #4	J/A-	
Hungary	Pigs	calicivirus	GII.11	1/17 (6)	RT-PCR	p290/p289	F	Farms #2	J/A+/-	[324]
	Dogs	calicivirus	GVI	2/63 (3)	RT-PCR	p290/p289	F	Shelter	NA	[325]
	Dogs	GIV.2	none	0/10 (0)	ELISA	VLPs of GIV.2 (GQ443611)	S	Clinics	J/A	[238]
	Lab mice	calicivirus	GV	10/41 (24)	RT-PCR	p289/p290, MNV specific primer set	PF	Outdoors	NA	[326]
	Mice[8]		GV	2/3 (67)						
	Mice[9]		none	0/7 (0)						
	Bank vole		none	0/3 (0)						

Location	Host	Investigated genogroup, genotype	Animal norovirus detected	Prevalence (%)	Detection method	Primers, probes and antigens used	Samples Type	Samples Location (n)	Age Clinical status	Ref
Africa										
Egypt	Cattle	GIII	GIII.2	6/25 (24)	RT-PCR	CBECU-F/CBECU-R	F	Farms #2	J+	[227]
Tunisia	Cattle	GIII	GIII.2	28/169 (17)	RT-PCR	CBECU-F/CBECU-R	F	Farms #17	J+	[228]
South Africa	Pigs	calicivirus	none	0/120 (0)	RT-PCR	p290/p289	RS	Farms #2	J	[256]
Ethiopia	Pigs	calicivirus	none	0/117 (0)	RT-PCR	p290/p110, G2SKF/ G2SKR	F	Households	J/A	[208]

[1] 64/312 (20%) in finisher pigs, 36/176 (20%) farms; [2] farms taken as n; [3] Cecum content; [4] House mouse (*Mus musculus*); [5] Wood mouse (*Apodemus sylvaticus*); [6] From carcass; [7] RNA from FFPE porpoise intestinal tissues; [8] Striped field mice (Apodemus agrarius); [9] Yellow - necked mice (Apodemus flavicollis); [10] Norway rat (*Rattus Norvegicus*); [11] Japanese field mouse (*Apodemus speciosus*); [12] Black rat (*Rattus rattus*); [*] Used same set of veal calfs'; [**] 9/45 (20) and 1/44 (2,3) in two different region.

Table S2. Studies investigating human-to-animal transmission and the detection methods that were applied. Included are studies that analysed fecal samples for human norovirus sequences by RT-PCR or real-time RT-PCR or serological studies. Some of the most frequently used primers are p289/p290 and p290/p110 (universal calicivirus primers, RdRp) and JV12/JV13 (norovirus specific). ELISA refers to serology ELISA. Unless otherwise stated sample were collected at one time point.
Sample type: F=Feces, FP=Pooled feces, S=Sera, IC=Intestinal content [a] longitudinal study; [b] case control; [c] outbreak.
Age, Clinical Status: A = adult (in pigs >6month), J = juvenile, +=symptomatic, -=asymptomatic

Location	Host	Investigated genogroup, genotype	Human norovirus detected	Prevalence (%)	Detection method	Primers, probes and antigens used	Samples Type	Samples Location (n)	Age Clinical status	Ref
The Americas										
USA	Pigs	GI.1, GII.4	GI.1	57/110 (63)	ELISA	VLPS of GI.1, GII.1	S	Farms Abattoirs #1	NA	[211]
			GII.4	47/110 (52)						
		calicivirus	none	0/117 (0)	RT-PCR	P289H,I/p290H,I,J,K	F/IC	Farms #1 Abattoirs #2	J-	
		GI, GII	NA	2/104	Antigen ELISA	Hyperimmune sera against GI.1, GI.2, GI.3, GII.1, GII.3, GII.4 and GII.9	F	Farms #1	J-	
	Pigs	calicivirus	none	0/9 (0)[1]	RT-PCR	p290/p110, G2SKF/ G2SKR	PF	Farms #9	A-	[193]
	Pigs	calicivirus	none	0/275 (0)	RT-PCR	p290/p110	F	Farms #6 Abattoirs #1	A-	[190]
	Pigs	calicivirus	none	0/139 (0)	RT-PCR	p290/p110	F	Farms #3	J	[296]
	Capuchin	GI.1, GII.4, GII.7	none	0/5 (0)	ELISA	VLPs of GI.1, GII.4, GII.7	S	Research institute #1	J/A-	[264]
	Mangabey		total	33/39 (85)						
			GI.1	29/39 (74)						
			GII.4	28/39 (72)						
			GII.7	15/39 (38)						

Location	Host	Investigated genogroup, genotype	Human norovirus detected	Prevalence (%)	Detection method	Primers, probes and antigens used	Samples Type	Samples Location (n)	Age Clinical status	Ref
USA	Pigtail	GI.1, GII.4, GII.7	total	11/17 (65)	ELISA	VLPs of GI.1, GII.4, GII.7	S	Research institute #1	J/A-	[264]
			GI.1	4/17 (24)						
			GII.4	4/17 (24)						
			GII.7	6/17 (35)						
	Rhesus		total	17/27 (63)						
			GI.1	11/27 (41)						
			GII.4	8/27 (30)						
			GII.7	4/27 (15)						
	Chimp		total	11/12 (92)						
			GI.1	11/12 (92)						
			GII.4	2/12 (17)						
			GII.7	0/12 (0)						
	Rhesus	calicivirus	Total	41/500 (8)	Real-time RT-PCR	Primers/probes specific for GI, GII, GIV	RS	Research institute #1	J+/-	[262, 263]
			GI.1	30/500 (6)						
			GII.7	8/500 (1,6)						
			GIV	3/500 (0,6)						
	Rhesus	GI.1	GI.1	114/188 (61)	ELISA	VLPs of GI.1, GII.5	S[a]	Research institute #1	J/A	[265]
		GII.5	GII.5	97/188 (52)						
	Cats	calicivirus	none	0/24 (0)	RT-PCR	p289/p290	F	Clinic #1 Shelter #1	J/A+/-	[240]
Canada	Pigs	calicivirus	none	0/20 (0)	RT-PCR	p289/p290	PF/F[2]	Farms #20, Abattoirs #2	J/A	[196]
	Pigs	norovirus	GII.4	4/120 (3)	RT-PCR	Region B and A primers, MON431-MON434, G1/G2	PF[a]	Farms #10	NA	[195]
	Cattle			1/179 (0,5)			PF	Farms #45	J/A	

Location	Host	Investigated genogroup, genotype	Human norovirus detected	Prevalence (%)	Detection method	Primers, probes and antigens used	Samples Type	Samples Location (n)	Age Clinical status	Ref
Venezuela	Pigs	calicivirus	none	0/137 (0)	RT-PCR	p289/p290, GLPSG1/ YGDDI1 GLPSG2/ YGDD1	F	Farms #7	J +/-	[204]
Nicaragua	Pigs	GII.4 Dijon	GII.4 Dijon	96/137 (70)	ELISA	VLPs of GII.4 (GU325839, AF472623), GII.3 (AY247431)[3]	S	Households	J/A	[257]
		GII.4 HS194	GII.4 HS194	94/137 (69)						
		GII.3	GII.3	80/137 (58)						
Brazil	Pigs	calicivirus	none	0/96 (0)	RT-PCR	p289/289HI, p290/290HIJK, Mon431/432/433/434	RS	Farms #5	J/A +/-	[301]
	Pigs	calicivirus	none	0/261 (0)	RT-PCR	289/289HI, 290/290HIJK, Cap C/D3/D1	F/IC	Farms #6, Abattoirs #1	J/A +/-	[302]
	Pigs	calicivirus	none	0/169 (0)	RT-PCR	p289/p290	RS	Farms #5#	J+/-	[303]
	Primates[4]	calicivirus	none	0/62 (0)	RT-PCR	p289/p290	F	Outdoors	-	[344]
	Cats	calicivirus	none	0/29 (0)	Real-time RT-PCR	p289H/p290H, FNOV-09/ FNOV-R15	F	Shelters #4	J+	[243]
Asia/New Zealand/Australia										
China	Pigs	calicivirus	none	0/209 (0)	RT-PCR	p289/p290	F	Farms #6	J/A-	[198]
	Pigs	calicivirus	none	0/904 (0)	RT-PCR	p289/p290	F	Farms #14	J/A	[200]
	Macaques[13]	calicivirus	GII.17	16/50 (32)	RT-PCR	GII.17 specific primers	F	Farm #1	NA	[260,261]
Taiwan	Pigs	calicivirus	GII.2/4	82/533 (32)	RT-PCR	p290/p110, NV2oF2/ NV2oR, G2F3/G2SKR	F	Farms #6	J/A-	[199]
Japan	Pigs	calicivirus	Total	11/354 (3)	RT-PCR, Real-time RT-PCR	p289/p290, G1SKF/ G1SKR G2SKF/G2SKR . Real-time RT-PCR primers: COG2F/ALPF/ COG2R	IC[a]	Farms #3 Abattoirs #1	A-	[201]
			GII.3	4/240						
			GII.4	7/240						
			GII.13	1/240						
	Pigs	SRSV	none	0/1117 (0)	RT-PCR	35/36 (SRSV), NV81/ NV82	IC[2]	Farms #26	-	[179]

Location	Host	Investigated genogroup, genotype	Human norovirus detected	Prevalence (%)	Detection method	Primers, probes and antigens used	Samples Type	Samples Location (n)	Age Clinical status	Ref
Japan	Pigs	calicivirus	none	0/24 (0)	RT-PCR	p289/p290	IC	Diagnostic #1	J+/-	[210]
	Dogs	caliciviurs	none	0/97 (0)	RT-PCR	p290d/p289d	F	Households	+	[242]
	Cats			0/83 (0)						
Korea	Pigs	GII	none	0/537 (0)	RT-PCR	GIIF2/GIIR (GII)	F	Farms #64	J/A +/-	[306]
	Pigs	Norovirus	none	0/576 (0)	RT-PCR	NORO-DG35OF/IF	F	Farms #12	NA	[202]
Iran	Cattle	GI, GII	NA	9/50 (18)	RT-PCR	Ni/E3	F	Farms #6	+	[231]
New Zealand	Pigs	GI-GIII	none	0/23 (0)	RT-PCR	GI-GIII primers	F	Farms #2	J-	[203]
	Sheep			0/33 (0)				Farms #2	J-	
	Cattle	GI-GIII	GIII.1	15/28 (54)	real-time RT-PCR	newly designed based on 52 norovirus sequences	F	Farms #2	-	[310]
Australia	Cattle	calicivirus	none	0/6 (0)	RT-PCR	p289/p290	PF	Farms	J-	[341]
Europe										
Italy	Pigs	calicivirus	none	0/290 (0)	RT-PCR	p290/p289/p110	F	Farms #17	J/A +/-	[288]
	Dogs	calicivirus	GIV.2	0/183 (0)	RT-PCR	p289/p290, JV12Y/JV13I	F	NA	J+	[312]
	Dogs	GII.4	GII.4	52/516 (10)	ELISA	VLPS of GII.4 (AY032605), GIV.1 (AF414427)	S	Households	J/A	[254]
		GIV.1	GIV.1	3/516 (0,6)						
	Dogs	calicivirus	none	0/239 (0)	RT-PCR	p298/p290, JV12/JV13	F	Households	J+/-	[236]
	Cats	calicivirus	none	0/105 (0)	RT-PCR	p298/p290, JV12/JV13	F	Shelters #3	J+/-	[241]
Spain	Pigs	calicivirus	NA	0/221 (0)	Real-time RT-PCR, RT-PCR	p289/p290, JV12/JV13, For real-time RT-PCR probes for GI and GII	F	Farms #14, Abattoirs #1	J/A+/-	[313]
	Dogs	calicivirus	none	0/26 (0)	RT-PCR	p298/p290, JV12/JV13	F	Households	J+/-	[236]
Portugal	Dogs	calicivirus	none	0/105 (0)	RT-PCR	p298/p290, JV12/JV13	F	Shelters, shops, clinics	+/-	[237]
France	Dogs	calicivirus	none	0/26 (0)	RT-PCR	p298/p290, JV12Y/JV13I	F	Households	J+/-	[236]

Location	Host	Investigated genogroup, genotype	Human norovirus detected	Prevalence (%)	Detection method	Primers, probes and antigens used	Samples Type	Samples Location (n)	Age Clinical status	Ref
Germany	Dogs	caliciviurs	none	0/3 (0)	RT-PCR	p298/p290, JV12Y/JV13I	F	Households	J+/-	[236]
	Norway rat[11,7]	all	GV	0/20 (0)	NGS		IC	outdoors	NA	[318]
Netherlands	Pigs	norovirus	none	0/100 (0)	RT-PCR, Southern blot	primers designed based on human norovirus	PF	Farms #100	A	[178]
	Cattle	norovirus	none	0/118 (0)			PF	Farms #118	J/A	
	Primates[7]	GI, GII	none	0/201 (0)	RT-PCR,	JV12/JV13	F	Research Institute #1	+/-	[268]
				0/158 (0)	ELISA	VLPs of GI.1, GII.4 (1995)	S	Research institute #1	NA	
	Chicken	norovirus	none	0/48 (0)	RT-PCR, Southern blot	primers designed based on human norovirus	PF	Broilers #48	J	[178]
Belgium	Pigs	calicivirus	none	0/43 (0)	RT-PCR	JV12/JV13, p289/p290	F	Diagnostic #1	J/A+/-	[287]
	Cattle	calicivirus	none	0/133 (0)	RT-PCR	JV12/JV13, p298/p290	F[a]	Diagnostic #1	J+	[223]
	Cattle	Norovirus	none	0/317 (0)	RT-PCR	JV12/JV13, CCV3/CCV4, CBECU-F/CBECU-R	F[a]	Farms	J/A+/-	[343]
UK	Cattle	GI, GII	none	0/476 (0)	RT-PCR	NI/E3 (GII), GLPSG1/ YGDD1 GLPSG2/YGDD1	F	Farms	J/A+	[345]
	Dogs	calicivirus	none	0/227 (0)	SYBR-based qPCR	p289/p290	F[b]	Clinics #4, Kennels	J/A+/-	[322]
	Dogs	GI, GII	none	0/248 (0)	Real-time RT-PCR	Primers to detect GI, GII	F[b]	Clinics #6, Shelter #1	J/A+/-	[255]
	Dogs	GI, GII	total	43/325 (13)	ELISA	Pooled VLPs for GI: GI.1, GI.2, GI.3 GII: GII.3, GII.4, GII.6, GII.12	S[b]	Kennel #1 veterinary college #1	NA	[255]
		GI	GI.1/2/3	8/325 (3)						
		GII	GII.3/4/6/ 12	20/325 (6)						
	Mice[5]	Norovirus	none	0/92 (0)	RT-PCR	general norovirus primers	F/RS[6]	Outdoors	NA	[323]
Ireland	Pigs	calicivirus	none	0/292 (0)	RT-PCR	p289/p290	F	Farms #4	J-	[205]

Location	Host	Investigated genogroup, genotype	Human norovirus detected	Prevalence (%)	Detection method	Primers, probes and antigens used	Samples Type	Samples Location (n)	Age Clinical status	Ref
Denmark	Rats[8]	calicivirus	GI.pb-GI.6	1/11 (9)	Real-time RT-PCR IEM	p289CVa/p289CVbp-289CVc/p289CVd	IC[6]	Sewersystem #5	NA	[267]
Finland	Dogs	GI, GII, GIV	GII.4	3/92 (3)	Real-time RT-PCR	COG2R/QNIF2 QNIFS for GII, in house primers and QNIF4/QNIF3 for GI, Mon4F/Mon4R and Ring4 for GIV	F	Household	J/A+/-	[253]
			GII.12	1/92 (1)						
	Rats[9]	GI, GII	NA	2/100 (2)	Real-time RT-PCR	GII: COG2R/QNIF2 and QNIFS (probe) GI: QNIF4/QNIF3 and JJV1P (probe)	F/IC[6]	Dump sites #3	NA	[266]
	Mice[10]			0/88 (0)				Outdoors		
	Birds	GI, GII	Total	31/115 (27)			F[12]	Dump sites #1		
			GII.4	4/115 (4)						
			GII.3	2/115 (2)						
			GII[11]	19/115 (17)						
			GI[11]	6/115 (5)						
Slovenia	Pigs	calicivirus	none	0/406 (0)	RT-PCR	p290/NVp110, JV12Y/JV13I JV12Y/NVp110 G1SKF/R G2SKF/R	F	Farms #8	J/A-	[207]
	Cattle			0/119 (0)			F	Farms #4	J/A-	
Hungary	Pigs	calicivirus	none	0/17 (0)	RT-PCR	p289/p290	F	Farms #2	J/A+/-	[324]
	Dogs	caliciviurs	none	0/63 (0)	RT-PCR	p289/p290	F	Shelter		[325]
	Rodents[8]	calicivirus	none	0/54 (0)	RT-PCR	p289/p290	PF	Outdoors	NA	[326]
Europe	Dogs	GI.1, GII.4	GI.1	9/308 (3)	ELISA	VLPs of GI.1 (NC_001959), GII.4 (JQ478409.1)	S	Clinics	J/A	[238]
			GII.4	106/308 (34)						

Location	Host	Investigated genogroup, genotype	Human norovirus detected	Prevalence (%)	Detection method	Primers, probes and antigens used	Samples Type	Samples Location (n)	Age Clinical status	Ref
Africa										
South Africa	Pigs	calicivirus	NA	1/120 (1)	RT-PCR	p289/p290	RS	Farms #2	J	[256]
Ethiopia	Pigs	calicivirus	GII.1	2/117 (2)	RT-PCR	p290/p110, G2SKF/ G2SKR	F	Households	J/A	[208]

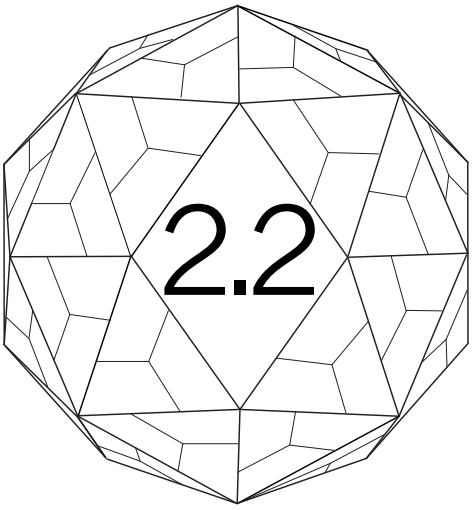
[1] Number of farms (n); [2] Cecum content; [3] From chronic patient; [4] Red-howler (*Alouatta seniculus*), Commo marmosets (*Callithrix jacchus*, free-ranging), Black-faced lion tamarin (*Leontopithecus caissara*); [5] *Mus musculus;* [6] From carcass; [7] Chimpanzees *(Pan troglodytes)*, Rhesus macaque (*Macaca mulatta*), Cotton top tamarins (*Saguinus Oedipus*), Cynomolgus macaque (*Macaca fascicularis*), Common marmoset; [8] Mice (*Apodemus agrarius*), Yellow necked mouse (*Apodemus flavicollis*), bank voles (*Myodes glareolus*); [9] Norway rat *(Rattus norvegicus);* [10]Yellow necked mouse (*Apodemus flavicollis*); [11] Could not be sequenced; [12] Collected from untouched fresh snow; [13] Rhesus/Pigtail/cynomologus.

Table S3. Summary of VLP attachment studies. Shown are the putative norovirus hosts for which attachment studies have been conducted. Their HBGA phenotype was extracted from papers that were chosen for the review. No additional papers were included.

Host	HBGA phenotype	VLPs (accession number)	Binding detected	No binding detected	Ref
Human	A+/B+/H1+/H2+/H3+/Lew^a+/Lew^b+/Lew^x+/Lew^y+	GII.4 (AAK84679) GII.5 (AF397156) GII.9 (AY038599)	PGM, PGIT	NA	[346]
		GI.1	PGM, PGIT[1,2] Epithelial cells of pig intestinal tissue	NA	
		GI.1 (JX023285)	Type II/III PGM[2]	NA	[347]
		GI.1, GI.3, GII.1, GII.3, GII.4	Porcine buccal and duodenal tissue[1]	GI.3 did not bind	[79]
		GI.1 NC_001959.2) GII.4 (AF472623)	Canine saliva/duodenal scrapings of secretors Canine duodenal tissue (antigen-A independent)	Human saliva of non-secretors	[255]
		GI.3 (KP064096) GII.3 (KP064097) GII.6 (KP064098) GII.12 (KP064099)	Canine duodenal scrapings of secretors	NA	[255]
		GII.4 (Dijon 171/96)	Human saliva of secretors	Bovine erythrocytes	[280]
		GII.4 (GU325839, AF472623), GII.3 (AY247431)	Type III PGM[1]	NA	[257]
		GII.1	NA	Bovine cell lines	[348]
		GI.1	Erythrocytes of chimpanzees	Erythrocytes of baboon, spider monkey, rhesus macaque, chicken, guinea pig, murine, canine, bovine, porcine, feline, and lapine	[349]
Pig	A+/H1+/H2+/Lew^b+ (PGM/PGIT, tissue)	GII.11	NA	52 human saliva (representing all major HBGA types)	[211]

Host	HBGA phenotype	VLPs (accession number)	Binding detected	No binding detected	Ref
Cattle	A+/B-/ H2+/ Lew^{y}+/ H1-/H3-/ Lew^{a}+[2] (fixed tissue)	GIII.2 (AF097917)	Bovine intestinal tissue (stomach, duodenum), bovine eryhthrocytes Bovine saliva Porcine intestinal tissue (non-epithelial cells) 2/44 synthetic glycans: (αGal-Lex, αGal tri, both have a terminal α1,3-galactose	Human saliva (A/B/O types)/ intestinal tissue 42/44 synthetic glycans tested	[280]
		GIII.2 (EU794907)	Bovine cell lines[4]	NA	[348]
Bat	NA	Bat norovirus (KJ790198)	H1, H3, A, B, α2,6-sialic acid (highest) (at 3 different temperatures)	H2, α-2,3-sialic acid Lew a,b,x,y	[248]
Rhesus macaques	A+/B+/ Lew^{b}+/ Lew^{x}+/ Lew^{y}+/ Lew^{a}- (saliva)	GII.17 (KX356908)[5]	Human saliva	NA	[260, 262, 263]
Dog	H2+/ A+/B-/ Lew^{y}+/ Lew_{b}-/ Lew^{x}- (saliva, intestinal scraping)	GVI.2 (GQ443611) GVI.1 (FJ692501) GIV.2 (EU224456.1)	Canine tissue (antigen-A independent)[1,6] 4/20 synthetic glycans: H1, A hepta, Lewb, Sial LNFV (1,2 fucose)	16/20 synthetic glycans	[170]
		GVI.2 (GQ443611)	Canine/human saliva of secretors	Human saliva of non-secretors	[170]
Cats	NA	GIV.2 (JF781268)	NA	30 synthetic glycans	[350]

[1] Blocking experiments were carried out; [2] VLP binding was abolished with KIO_4 pre-treatment (oxidizes carbohydrates); [3] only in goblet cells of duodenum and colon; [4] Madine-darby kidney cells (MDBK), bovine turbinate cells, embryonic bovine tracheal cells, Georgia bovine kidney cells, embryonic bovine lung cells, MacT-cells (udder origin), bronchial cells, Bomac cells (macrophagic origin), jejunocytes; [5] Isolated from rhesus macaques; [6] Pre-treatment with 1,2α-fucosidase diminished VLP binding to tissue (removes the fucose group from the H antigen). PGIT=porcine gastrointestinal tissue washing, NA=not applicable. PGM=porcine gastric mucin



Human norovirus at the human-animal interface

Phylogenetic investigation of norovirus transmission between humans and animals

Nele Villabruna, Ray W. Izquierdo-Lara, Judit Szarvas, Marion P.G. Koopmans and Miranda de Graaf

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Abstract: Norovirus infections are a leading cause of acute gastroenteritis worldwide, affecting people of all ages. There are 10 norovirus genogroups (GI-GX) that infect humans and animals in a host-specific manner. New variants and genotypes frequently emerge, and their origin is not well understood. One hypothesis is that new human infections may be seeded from an animal reservoir, as human noroviruses have occasionally been detected in animal species. The majority of these sequences were identified as older GII.4 variants, but a variety of other GIIs and GIs have been detected as well. While these sequences share at least 94% nt similarity with human strains, most of them are >98% identical to human strains. The fact that these strains were detected in animals after they had been detected through human surveillance to be already circulating in humans suggests human-to-animal transmission.

1. Introduction

Noroviruses are an important cause of gastroenteritis in humans and animals [172]. Their genome is 7.5 kb in length and organized in three open reading frames (ORF1-3) [12]. ORF1 encodes a polyprotein that is enzymatically cleaved by the viral protease into six proteins, including RNA-dependent RNA polymerase (RdRp). ORF2 and ORF3 encode for the major and minor capsid protein (VP1 and VP2), which make up the virus capsid. VP1 is composed of the conserved shell-domain and the protruding (p) domain, which contains the receptor binding sites that recognize histo-blood group antigens (HBGAs), and the antigenic sites [351, 352]. Based on phylogenetic analysis of VP1 sequences, 10 genogroups have been identified (GI-GX), which are further divided into 49 genotypes, of which some include several variants [3]. Viruses within genogroups I, II, IV, VIII, and IX infect humans, with GI and GII being the most commonly detected genotypes. Viruses from the other genogroups have been found in a broad range of animals including cattle and sheep (GIII), cats and dogs (GIV, GVI, and GVII), rodents (GV), bats (GX), and harbor porpoises (GNA1). Despite this large number of genotypes, viruses within GII.4 are most commonly detected in humans and are responsible for the majority of outbreaks [31, 38, 353]. Norovirus diversity is additionally increased by recombination events between ORF1 and ORF2, resulting in new strains. New variants, genotypes, and recombinants frequently emerge in the human population, yet their origin is unknown. One hypothesis is that they originate from an animal reservoir. We have previously systematically reviewed serological evidence of transmission between animals and humans and described that more evidence exists for human-to-animal transmission than vice versa [354]. However, given the presence of host-specific noroviruses, the possibility of serological reactivity due to the presence of cross-reactive antibodies cannot be excluded. More conclusive evidence can be gained from virological testing, and although viral RNA of animal strains has not been detected in humans, viral RNA of human GI and GII strains has been detected in fecal material of calves, pigs, birds, captive macaques, dogs, and

rodents (reviewed in reference [354]). Most of these animals are also susceptible to human noroviruses under experimental conditions [51]. This implies that animals could be a possible reservoir for human noroviruses. To explore this possibility and investigate the genetic relationship of human noroviruses detected in animals and humans, we have analyzed all human norovirus sequences that, to date, have been found in animal stool samples.

2. Results

2.1. Norovirus strains, closely related to human noroviruses, are found in animals

Published sequences of human noroviruses detected in animal feces were collected [354], and sample information is summarized in **Table 1**. Human noroviruses have been found in a variety of mostly asymptomatic animals, of which the domestic pig was the most common species. While three whole genomes have been sequenced (two GII.4 Sydney[P31] from dogs and one GII.17[P17] from a rhesus macaque), most published sequences are short, 200–300 bp in length, and cover the 5' end of VP1, reflecting commonly used targets for diagnostic RT-PCR assays. Single sequences that cover different parts of ORF1 were not used for phylogenetic analysis but are listed in **Table 1**.

Overall, the animal strains are very close or even identical to human strains, ranging from 94% to 100% nt identity. It is worth noting that none of these strains differed enough to be categorized as a new variant. Three sequences belonged to the GI genogroup and all others to GII, GII.4 being the most commonly found genotype. Most GII.4 sequences were typed as older variants, predominantly den Haag 2006b, but also Farmington Hills 2002 (only RdRp), Asia 2003, Yerseke 2006a, Apeldoorn 2007, New Orleans 2009, and more recently GII.4 2012 Sydney (**Fig. 1**). The isolation dates of these samples coincide with the end of the time period that these strains were circulating in the human population (**Table 1**). Den Haag 2006b was most prevalent in the human population between 2006 and 2008 [22], but the collection dates of the animal samples fell between 2008 and 2009, with the exception of one RdRp sequence, which was collected in 2005. Two studies which included samples of close contact humans with symptoms detected identical GII.4 sequences in dogs and their owners: the two full genome GII.4 Sydney sequences found in Thailand and an unassigned GII.4 in Finland [253, 355]. Most studies, unfortunately, did not include samples of close contact humans.

Table 1. Information about human norovirus sequences found in animals.

Accession number	Host	Location	Year	Circulation Based on References [22, 177]	Norovirus Typing	Sequence Length (bp)	Genome region covered	Similarity to best BLAST hit	Ref
MF444290	Bird	Finland	2009	NA	GII.4 could not assign	223	VP1	222/223 (99.5%)	[266]
MF444291	Bird	Finland	2009	NA	GII.4 could not assign	223	VP1	222/223 (99.5%)	[266]
MF444292	Bird	Finland	2009	2006-8	GII.4 Den Haag 2006b	223	VP1	222/223 (99.5%)	[266]
MF444293	Bird	Finland	2011	2009-12	GII.4 New Orleans 2009	223	VP1	223/223 (100%)	[266]
JQ068133 [1]	Pig	Taiwan	2008	2006-8	GII.4 Den Haag 2006b	239	RdRp/VP1	239/239 (100%)	[199]
AB521758-63	Pig	Japan	2008/9	2006-8	GII.4 Den Haag 2006b	302	VP1	298/302 (98.9%)-302/302 (100%)	[201]
AB521764	Pig	Japan	2009	2002-6	GII.4 Asia 2003	302	VP1	302/302 (100%)	[201]
JF746890 [2]	Dog	Finland	2009	NA	GII.4 could not assign	283	VP1	281/283 (99.3%)	[253]
JF746891	Dog	Finland	2010	NA	GII.4 could not assign	283	VP1	281/283 (99.3%)	[253]
JF746892	Dog	Finland	2009	2006-8	GII.4 Den Haag 2006b	283	VP1	281/283 (99.3%)	[253]
MK928498–99 [2]	Dog	Thailand	2018	2012-20	GII.4 Sydney 2012[P31]	7513	Genome	7457/7513 (99.3%)	[355]
EF175441 [7]	Pig	Canada	2005	2002-4	GII.P4 Farmington Hills 2002	172	RdRp	172/172 (100%)	[195]
CE-M-05–0114[6,7]	Pig	Canada	2005	NA	GII could not assign	172	RdRp	172/172 (100%)	[195]
CE-M-06–0013[6,7]	Pig	Canada	2005	2002-4	GII.P4 Farmington Hills 2002	172	RdRp	169/172 (98.3%)	[195]
CE-M-06–0509[6,7]	Cattle	Canada	2005	2006-8	GII.P4 Den Haag 2006b	172	RdRp	168/172 (98.3%)	[195]
GU556160-66[7]	Pig	Taiwan	2008	2006-8	GII.P4 Den Haag 2006b	260	RdRp	241/256 (94%)	[199]
MN175616	Bird	Brazil	2016	NA	GI.2	492	VP1	475/478 (99.4%)	[366]
KT943503	Macaque[3]	USA	2008	NA	GI.1	1670	VP1-VP2	1670/1670 (100%)	[263]
KC294198	Rat[4]	Denmark	2012	NA	GI.6[GI.Pb]	4012	RdRp-VP1/2	3959/3993 (99.1)	[267]
MF444294	Crow[5]	Finland	2011	NA	GII.3	223	VP1	223/223 (100%)	[266]
KX073992	Pig	Ethiopia	2013	NA	GII.1	291	VP1	279/291 (95.9%)	[208]
KT943504	Macaque[3]	USA	2008	NA	GII.7	1623	VP1	1570/1632 (96%)	[263]
KT943505	Macaque[3]	USA	2008	NA	GII.7	1623	VP1	1570/1632 (96%)	[263]
JF746893	Dog	Finland	2009	NA	GII.12	283	VP1	282/283 (99.7%)	[253]
KX356908	Macaque[3]	China	2015	2014-15	GII.17[P17]	7556	Genome	7496/7556 (99.2%)	[261]
AB521765	Pig	Japan	2008	NA	GII.14	302	VP1	291/302 (96.4%)	[201]
AB521754-57	Pig	Japan	2008	NA	GII.3	302	VP1	297/302 (98.3%)-301/302 (99.7%)	[201]
JQ068117-132	Pig	Taiwan	2008	NA	GII.2	239	VP1	238/239 (99.6%)	[199]
HM035148[7]	Macaque[3]	USA	2008	NA	GII.7 could not assign[8]	274	RdRp	259/274 (94.5%) to GII.P7	[262, 263]
MN175617[7]	Bird	Brazil	2016	NA	GII.P31 could not assign[8]	438	p48	416/419 (99,3%) (gap)	[366]

[1] Was not included in phylogeny because coverage of VP1 was too short for alignment; [2] Had identical nt with sequences found in associated human; [3] *Macaca mulatta*; [4] *Rattus norvegicus*; [5] *Corvus corone cornix*, for other birds no more details were given; [6] Sequence was only published in paper but without accession number; [7] Were not included because they covered regions outside ORF2. [8] Outside typing region. Genotype based on closest blast hit. VP1=Virus capsid protein 1 (ORF2), VP2=Virus capsid protein 2 (ORF3), RdRp=RNA-dependent RNA polymerase.

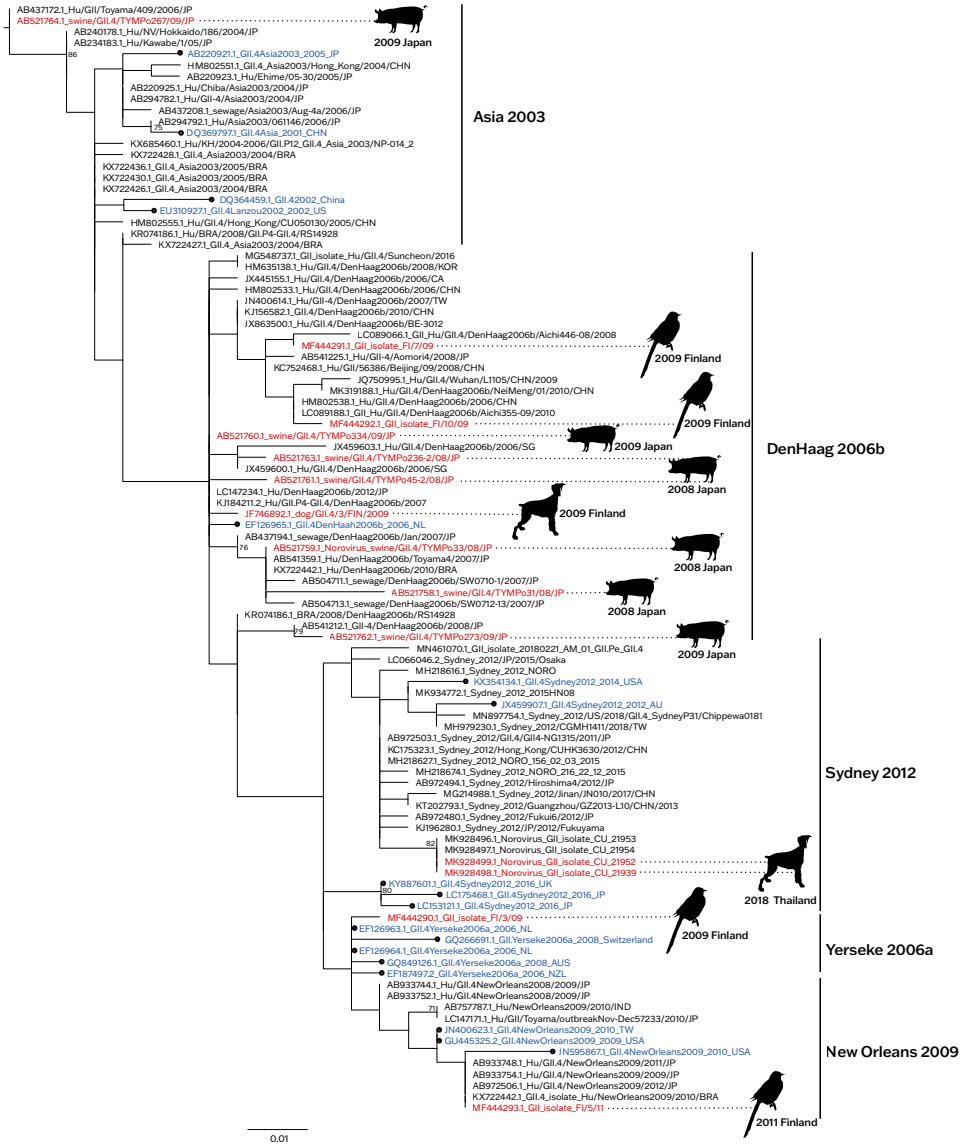


Figure 1. Genetic characterization of GII.4 sequences found in animals. A maximum-likelihood tree based on 223 bp GII.4 sequences was inferred with PhyML v3.0 software using the general time reversible nucleotide substitution model (GTR + G). Sequences that were found in animals (**red**) were aligned with most closely related human sequences (**black**) and the reference sequences from the NoroNet typing tool (**blue**, **black circle**). The animal in which norovirus was found as well as the date and country of collection are indicated next to the sequence. The scale bar indicates nucleotide substitutions per site.

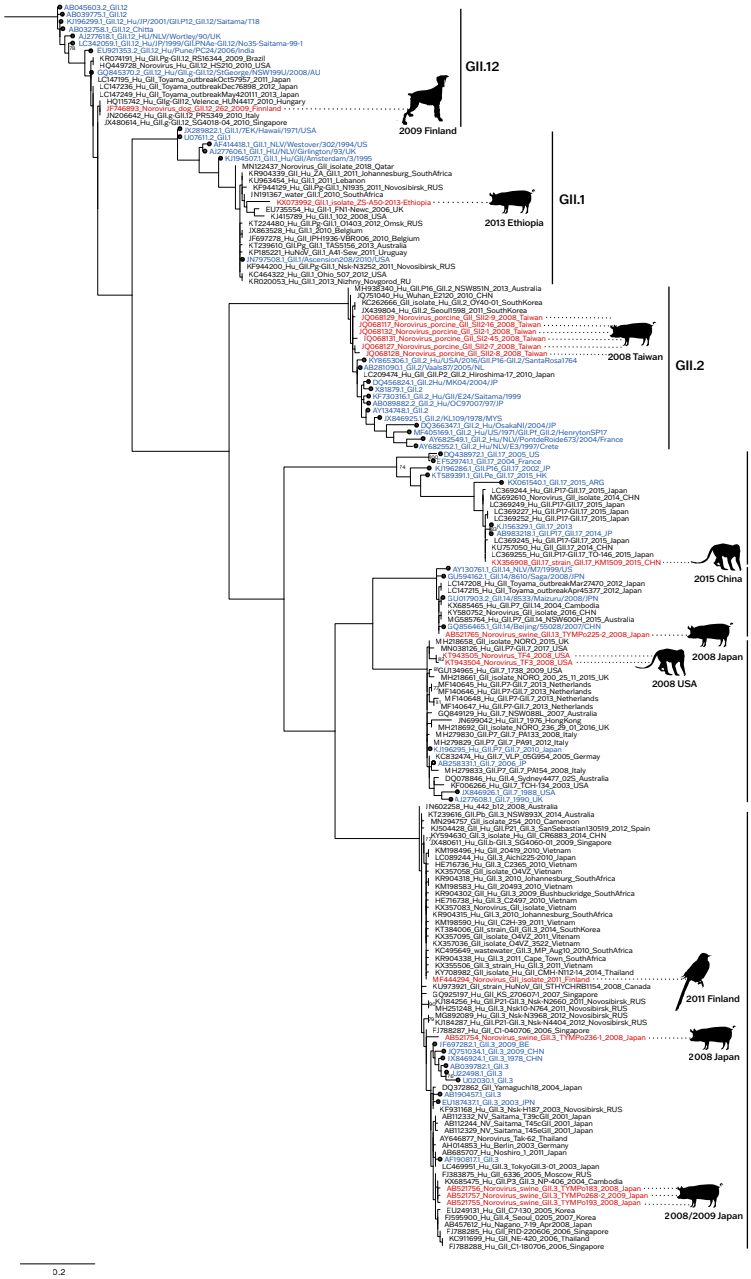


Figure 2. Genetic characterization of non-GII.4 GII sequences found in animals. A maximum-likelihood tree based on 180 bp GII sequences was inferred with PhyML v3.0 software using the general time reversible nucleotide substitution model (GTR + G). Sequences that were found in animals (**red**) were aligned with most closely related human sequences (**black**) and the reference sequences from the NoroNet typing tool (**blue**, **black circle**). The animal in which norovirus was found as well as the date and country of collection are indicated next to the sequence. The scale bar indicates nucleotide substitutions per site.

Figure 3. Genetic characterization of GI sequences found in animals. A maximum-likelihood tree based on 271 bp GI sequences was inferred with PhyML v3.0 software using the general time reversible nucleotide substitution model (GTR + G). Sequences that were found in animals (**red**) were aligned with most closely related human sequences (**black**) and the reference sequences from the NoroNet typing tool (**blue**, **black circle**). The animal in which norovirus was found as well as the date and country of collection are indicated next to the sequence. The scale bar indicates nucleotide substitutions per site.

While GII.4 was the most commonly found genotype, other GII and GI noroviruses have been detected as well (**Fig. 2** and **Fig. 3**). Some strains matched the then-circulating strains in the human population, such as GII.3 and GII.17, of which the latter was one of the most prevalent genotype in the period 2014–2015 [259]. Other strains are less frequently found in humans, and their discovery in animals was therefore more surprising. These include the GI genotypes as well as GII.1, GII.2, GII.12, and GII.14 viruses. The recent finding of a GI.1 virus, which was identical to the prototype strain first isolated in 1968, is unexpected. This specific GI.1 is not detected in humans anymore, but newer GI.1 variants are sporadically detected in humans and in sewage [31, 356, 357]. These findings spark the question of whether the less frequently detected GII and GI viruses continue to circulate undetected in humans and animals.

2.2. Molecular clock phylogeny of GII.7 and GII.17 genotypes

To investigate the evolutionary relationship between noroviruses detected in humans and animals and to estimate how long ago they diverged, we conducted a BEAST analysis. Of the noroviruses found in animals, the complete VP1 sequences were only available for viruses belonging to GII.4 (MK928498-99), GI.1 (KT943503), GI.6 (KC294198), GII.17 (KX356908), and GII.7 (KT943504/5). Of these, two GII.7 sequences and a GII.17 sequence (all found in rhesus macaques) were the only sequences with nonsynonymous mutations compared to the most closely related human strains. To determine the time to the most recent common ancestor (tMRCA) of the rhesus macaque-derived VP1 gene sequences to those found in humans, we performed separate BEAST analysis for these two genotypes. The estimated time to the MRCA of the rhesus macaque GII.7 to known human strains was shown to be around the end of the year 2000 (between 1998.0 and 2003.8, 95% HPD), eight years before the rhesus macaque GII.7 was detected (**Fig. 4A**). For the rhesus macaque GII.17 this was around 2011 (between 2010.3 and 2012.7, 95% HPD), four years before the rhesus macaque GII.17 strain was detected, and the tMRCA predated the GII.17[P17] outbreaks in humans during the winter of 2014–2015 (**Fig. 4B** and **Fig. S1**). However, it should be noted that the tMRCA 95% HPD interval is large and does not necessarily predate the tMRCA solely human GII.17 strains within the same clade.

2.3. Animal GII.7 and GII.17 sequences contain amino acid changes that are located either in or adjacent to antigenic epitopes

Amino acid changes in the exposed protruding p domain of the capsid can lead to differences in either HBGA binding or antigenic drift [4]. To identify whether the 13 and 4 amino acid changes found in GII.7 and GII.17 VP1 sequences from macaques are close to an antigenic epitope or receptor binding site, we mapped their location onto the predicted 3D GII.7 structure of the P domain and a 3D GII.17 P domain structure, respectively. The antigenic epitopes were predicted based on an alignment with GII. 4 sequences. The GII.7 sequence had several amino acid changes that were located either within a predicted antigenic epitope or in close proximity (**Fig. 5A**). Three changes were located directly in the predicted antigenic epitopes (**Fig. 5C** and **Fig. S2**): N294S and G295V in epitope A, and N346I in epitope C. Another seven were in close proximity to predicted epitopes: E375G was situated right next to the HBGA binding site, N343G, V290I and I291T were adjacent to epitope C, and V404A, R401Q, and L446M were next to epitope D. Two changes, I478V and Y514H, were on the surface but distant from any epitopes, while T54N was located outside of the P domain. Of the four changes found in the GII.17 sequence, N342S was the only one in proximity to epitope C (**Fig. 5B** and **C**, **Fig. S2**). Y505H was on the surface but distant from any predicted epitopes. P280S and G282D are not surface exposed. Thus, some of the observed mutations potentially affect HBGA-binding specificity and antigenicity.

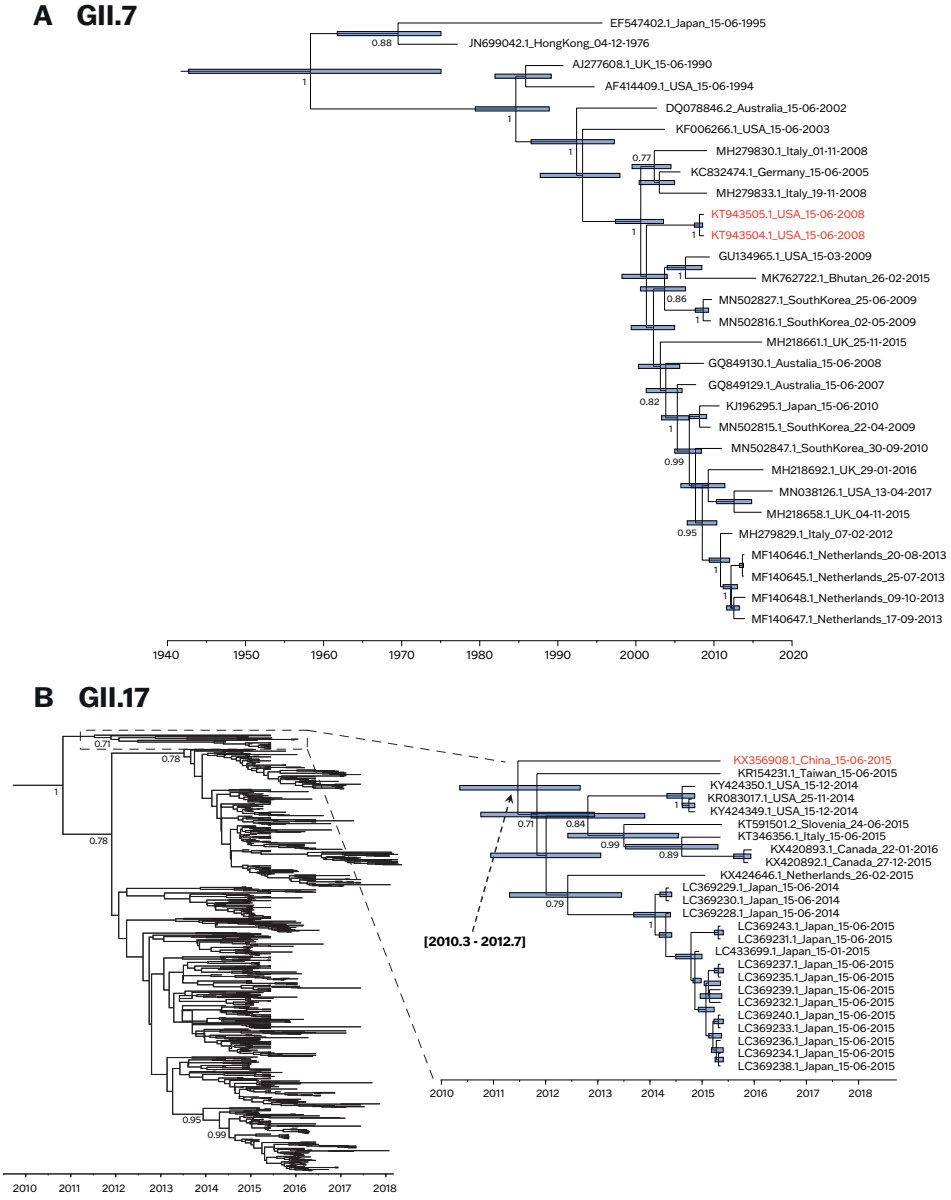
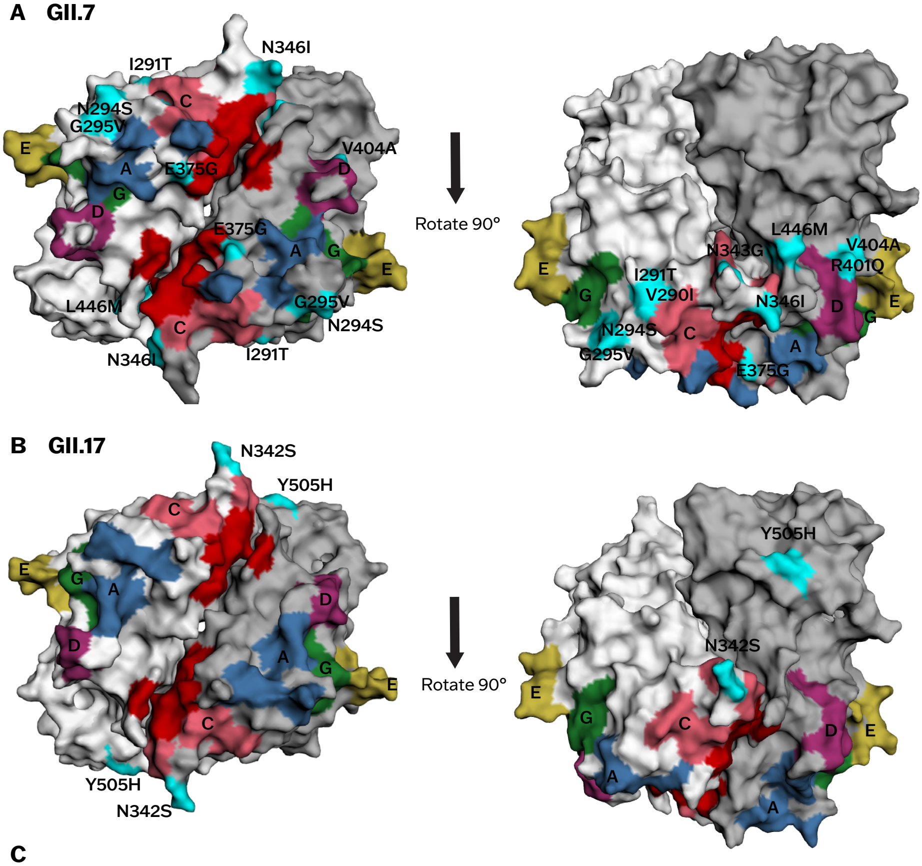


Figure 4. Molecular clock phylogeny of the complete VP1 gene sequences. (**A**) For GII.7 and (**B**) for GII.17 (Kawasaki308 cluster) constructed by the Bayesian Markov chain Monte Carlo (MCMC) method. Sequences in red indicate sequences from animal origin. Node bars indicate the 95% HPDs of the time of the most common recent ancestor. Numbers in the nodes show the posterior densities (only values >0.7 are shown). The expanded GII.17 tree is shown in **Fig. S1**.



C

Mutations in antigenic epitopes

Epitopes	A								C						
aa*	294	295	296	297	298	368	372	373	339	340	341	375	376	377	378
GII.4 2004	A	G	T	Q	N	S	S	N	R	R	D	F	E	T	G
GII.17	I	N	R	D	R	R	-	N	V	A	P	D	F	Q	F
GII.17 rhesus	I	N	R	D	R	R	-	N	V	A	P	D	F	Q	F
GII.7	N	G	S	Y	R	F	-	Q	K	N	P	F	E	V	H
GII.7 rhesus	S	V	S	Y	R	F	-	Q	K	I	P	F	E	V	H

Epitopes	D						E					G					
aa*	391	393	394	395	396	397	407	411	412	413	414	352	355	356	357	359	364
GII.4 2004	D	S	T	T	H	Q	N	R	D	S	H	S	S	V	H	T	S
GII.17	D	D	G	H	P	F	N	E	L	T	L	S	S	P	Q	V	S
GII.17 rhesus	D	D	G	H	P	F	N	E	L	T	L	S	S	P	Q	V	S
GII.7	D	S	-	H	P	F	R	H	L	T	N	N	G	D	Q	A	Q
GII.7 rhesus	D	S	-	H	P	F	R	H	L	T	N	N	G	D	Q	A	Q

Figure 5. Mapping of amino acid differences between human strains found in animals versus humans onto p-dimers (the two subunits are shown in two shades of **grey**, top-view). Amino acid changes exclusively found in the strains detected in animals are colored in **turquoise**. Predicted HBGA binding sites are marked in **red**, and the epitopes A-G in color. Changes were mapped onto the P domain of (**A**) GII.7 and (**B**) GII.17. The 3D structures were predicted by SWISS-MODEL on the basis of the crystal structure of the P domain of a GII.17 strain (PDB number 5f4o.1) for GII.7 (KT943504 and KT943505) and GII.17 Kawasaki (5LKG) for GII.17 (KX356908). (**C**) The alignment was performed with ClustalW and shows the amino acids defining the antigenic sites that differ between strains found in animals and humans. *Amino acid numbering was based on GII.4 2004. Putative binding sites were taken from reference [274].

3. Discussion

Norovirus genome sequences that are very similar or even identical to those of human strains have been detected in animals all over the world. The timing of detection of human-like sequences in animals almost invariably coincides with the circulation of the matching variants in the human population, and most sequences were highly similar, indicating a recent spillover. This was especially visible for GII.4 viruses, which—in the human population—undergo epochal evolution leading to emergence of antigenically distinct variants every few years, replacing the previous viruses [177]. For the GII.4 viral genomes detected in animals, assuming that the direction of transmission was from humans to animals seems most plausible, as the GII.4 variants were circulating in humans before they were found in animals. This was also the case for two studies that analyzed human and animal virus sequences from the same household [253, 355].

The epidemiology of non-GII.4 genotype noroviruses is distinct. Non-GII.4 viruses also have a global distribution, and cause sporadic infections and outbreaks [31], but do not evolve as rapidly as GII.4 variants and do not show the pattern of variant replacement [4]. Nevertheless, our analysis showed that most GII sequences found in animals were also very close or identical to human strains, arguing against long-term circulation in animals. It should, however, be noted that sequence information was often limited to very short fragments that are commonly used as diagnostic targets, as the sequences cover conserved regions. It is intriguing that the two longer sequences belonging to GII.7 and GII.17 that were available were the viruses with the most diverged nucleotide sequence compared to human variants. They were both found in captive macaques, but no information about humans or contaminated food from those centers was available. The BEAST analysis placed the most recent common ancestor to human isolates four and eight years before their detection in macaques, revealing a considerable temporal and genetic gap of these genotypes. For the GII.17[P17] strain detected in macaques the tMRCA predated their detection in humans. This can be explained either by lack of knowledge about the GII.7 and GII.17 diversity in humans or by the undetected circulation of these genotypes in a non-human reservoir. GII.7, and to a lesser degree GII.17, had accumulated amino acid changes that were located in regions predicted to define antigenicity of norovirus, thereby possibly resulting in an adapted phenotype. The epitopes in GII.7 and GII.17 were inferred from those of GII.4. It should be noted that these have only been established as antigenic epitopes in GII.4 and not for any other genotype. However, comparison of capsid sequences indicates that GII.17 is evolving at previously defined GII.4 antibody epitopes [358]. In our analysis, the rhesus macaque GII.17 strain only had one mutation near the HBGA binding site compared to the most closely related strains detected in humans. Saliva binding studies using recombinant protein showed that the rhesus macaque GII.17 strain binds to human saliva samples with significantly lower binding signals than a

similar human GII.17 strain with two mutations near the HBGA binding site [261]. Thus, animals can harbor human norovirus strains that potentially have antigenic and binding properties that differ from those detected in humans.

As the interface between wildlife and domesticated animals and humans is expanding, the risk of pathogens jumping the species barrier increases. While much of current virus research is focused toward transmission from animals-to-humans, our results show that the reverse should not be neglected, as it might have consequences for pathogen dynamics in humans as well as in animals. How often human-to-animal transmission of norovirus occurs, and if they are single events or if human strains circulate continuously in some animal reservoir, needs to be further addressed. Given the prevalence of host-specific viruses in several of the species of animals in which human norovirus sequences were detected, there is at least in theory the potential for recombination in case of dual infections. The question of whether human noroviruses in animals or recombinant human animal norovirus genomes are transmitted back into the human population, and therefore have an impact on (re)-emergence of noroviruses, remains to be answered.

4. Materials and Methods

4.1. Phylogenetic analyses

Published sequences of human noroviruses detected in animal feces were collected and searched against the entire GenBank database for DNA sequence (BLASTN). The 20 best hits were downloaded and typed using the NoroNet typing tool [174]. Blast hits that were identical to each other were excluded. Sequences from animal inoculation experiments were also excluded. For the phylogeny we used the blast hits as well as sequences of the respective genotypes and variants from the NoroNet typing tool reference sequence set (https://www.rivm.nl/mpf/typingtool/norovirus). Alignments were made using MUSCLE [359]. Maximum likelihood trees were created with PhyML v3.0 [360] (http://www.atgc-montpellier.fr/phyml/) and an automated model was selected by Smart Model Selection (SMS [361]) with 100 bootstrap replications. The trees were visualized using FigTree v1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/).

4.2. BEAST analyses

GII.7 and a GII.17 were the only sequences of which the whole VP1 was available and which contained nonsynonymous mutations compared to the most closely related human strains. Therefore, these were used in the BEAST analysis. All complete or near complete GII.7 and GII.17 VP1 sequences were downloaded from GenBank and aligned separately with MUSCLE [359]. The temporal signal of each group of sequences was evaluated with TempEst v1.5.3 [362] and sequence outliers were

removed from the final dataset. Bayesian phylogenetic trees based on complete VP1 sequences were inferred using BEAST v1.10.4 [363]. For GII.7 sequences, the final dataset included 29 sequences in the alignment (1560 bp). The general time reversible (GTR) substitution model was used with 4 gamma categories with 3 partitions into codon positions to generate an uncorrelated relaxed molecular clock. The tree prior was set as an exponential growth and random sampling. The Markov chain Monte Carlo (MCMC) was set to 50,000,000 generations to ensure convergence. For GII.17 sequences, the final dataset included 764 sequences in the alignment (1484 bp), corresponding to the period between 2013 and 2018 and belonging to the Kawasaki308 cluster. The HKY substitution model and the population size was assumed to be constant throughout its evolutionary history. The MCMC run was set to 400,000,000 generations to ensure convergence. In both datasets, if the day from the collection date was missing, the day was set as the 15th of the given month. If both day and month were missing, the collection date was set as June 15 of the given year. Log files were analyzed in Tracer v1.7.1 to check if ESS values were beyond threshold >200 [364]. The maximum clade credibility tree was constructed with 10% burn-in of the trees using TreeAnnotator v1.10.4. Trees were annotated and visualized using FigTree v1.4.3. The reliability of the branches was supported by 95% highest posterior densities (HPDs).

4.3. Mapping of amino acid changes onto 3D structure

Amino acid changes of GII.7 and GII.17 that were unique to strains found in animals, were mapped onto 3D P particle structures using EzMol v2.1 [365]. The three-dimensional structure of the GII.7 strain was predicted by homologous modelling using SWISS-MODEL server (available at https://swissmodel.expasy.org/interactive) with default settings. The model was built on the basis of the crystal structure of the P domain of a GII.17 strain (PDB number 5f4o.1). For GII.7 (KT943504 and KT943505) the predicted 3D P domain structure was used and for GII.17 (KX356908) the P domain structure of GII.17 Kawasaki (5LKG). The antigenic epitopes were inferred from those of GII.4 using multiple sequence alignment and information on the HBGA binding site was taken from reference [274].

Supplementary Materials: The following are available online at http://www.mdpi.com/1999-4915/12/11/1287/s1, Figure S1: Extended tree of molecular clock phylogeny of the complete VP1 gene sequences for GII.7, Figure S2: Multiple amino acid sequence alignment of the norovirus GII.7 and GII.17 P domain.

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Author Contributions: Conceptualization, N.V., R.W.I.-L., and M.d.G.; methodology, N.V., J.S., R.W.I.-L., and M.d.G.; formal analysis, N.V. and R.W.I.-L.; writing, original draft preparation, N.V. and R.W.I.-L.; writing, review and editing, M.P.G.K. and M.d.G.; supervision, M.P.G.K. and M.d.G.; funding acquisition, M.P.G.K. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

Supplementary material

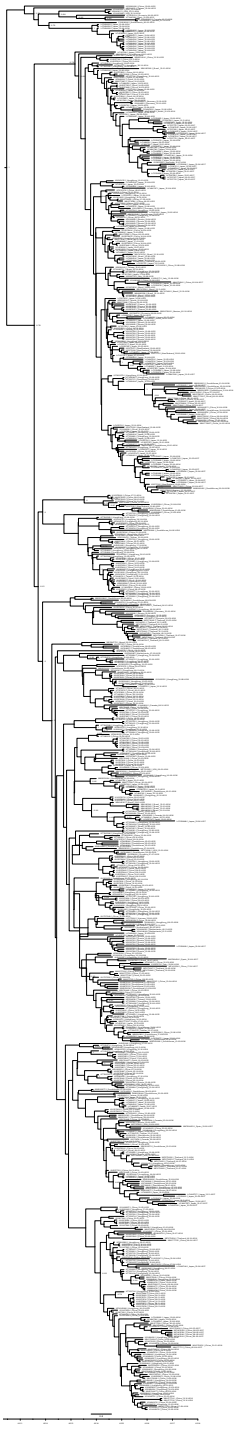


Figure S1. The extended molecular clock phylogeny of the complete VP1 gene sequences for the GII.17 (Kawasaki308 cluster) constructed by the Bayesian MCMC method. Numbers in the nodes show the posterior probabilities (only values >0.7 are shown). The scale bar indicates nucleotide substitutions per site.

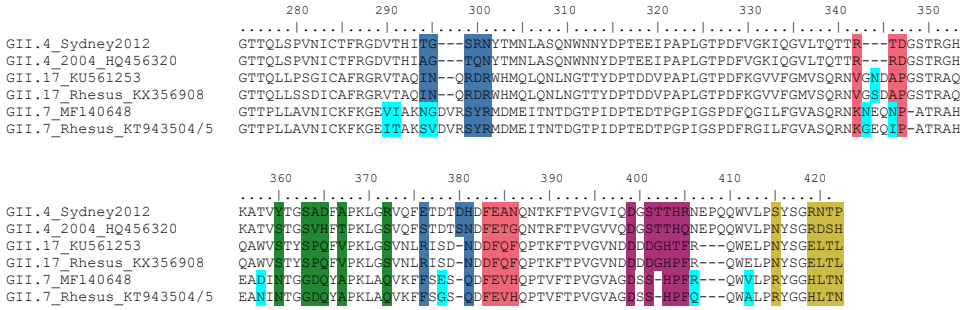
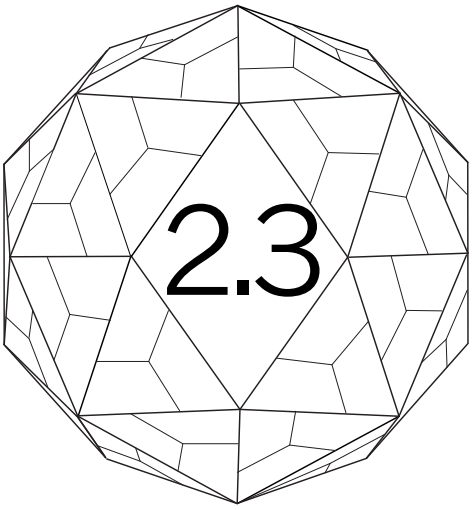


Figure S2. Multiple amino acid sequence alignment of the norovirus GII.7 and GII.17 P domain. Positions of GII.4 antigenic epitopes A–G are colored in the same code as in **Fig. 5**.



Human norovirus at the human-animal interface

Human noroviruses attach to intestinal tissues of a broad range of animal species

Nele Villabruna, Claudia M.E. Schapendonk, Georgina I. Aron, Marion P.G. Koopmans and Miranda de Graaf

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Abstract: Human noroviruses are the most common nonbacterial cause of gastroenteritis outbreaks, with new variants and genotypes frequently emerging. The origin of these new viruses is unknown; however, animals have been proposed as a potential source, as human noroviruses have been detected in animal species. Here, we investigated the potential of animals to serve as a reservoir of human noroviruses by testing norovirus attachment to formalin-fixed intestinal tissues of a range of potential reservoir animals. We set up a novel method to study norovirus binding using fluorescein isothiocyanate (FITC)-labeled virus-like particles (VLPs). In humans, noroviruses interact with histo-blood group antigens (HBGAs), carbohydrates that are expressed, among others, on the epithelial lining of the gastrointestinal tract. In animals, this interaction is not well understood. To test if virus binding depends on HBGAs, we characterized the HBGA phenotype in animal tissues by immunohistochemistry. With the exception of the black-headed gull and the straw-colored fruitbat, we observed the attachment of several human norovirus genotypes to the intestinal epithelium of all tested animal species. However, we did not find an association between the expression of a specific HBGA phenotype and VLP attachment. We show that selected human noroviruses can attach to small-intestinal tissues across species, supporting the hypothesis that human noroviruses can reside in an animal reservoir. However, whether this attachment can subsequently lead to infection needs to be further assessed.

Importance: Noroviruses are a major cause of acute gastroenteritis in humans. New norovirus variants and recombinants (re)emerge regularly in the human population. From animal experiments and surveillance studies, it has become clear that at least seven animal models are susceptible to infection with human strains and that domesticated and wild animals shed human noroviruses in their feces. As virus attachment is an important first step for infection, we used a novel method utilizing FITC-labeled VLPs to test for norovirus attachment to intestinal tissues of potential animal hosts. We further characterized these tissues with regard to their HBGA expression, a well-studied norovirus susceptibility factor in humans. We found attachment of several human strains to a variety of animal species independent of their HBGA phenotype. This supports the hypothesis that human strains could reside in an animal reservoir.

1. Introduction

Noroviruses are an important cause of gastroenteritis in humans and animals. To date, 10 genogroups (G) have been identified (GI to GX), which are further divided into 49 genotypes [3]. Viruses within genogroups GI, GII, GIV.1, GVIII, and GIX are known to infect humans, while viruses from other genogroups have been found in a range of animals: pigs (GII.11, GII.18, and GII.19), cattle (GIII.1 and GIII.2), sheep (GIII.3), rodents (GV.1 and GV.2), cats (GIV.2, GVI.1, and GVI.2), lions (GIV.2), dogs (GVI.1, GVI.2, and GVII), harbor porpoises (GNA1), sea lions (GNA2), and bats (GX). Based on the whole capsid protein, viruses of different genogroups share 50% amino acid identity, while genotypes within the same genogroup share 60% amino acid identity. Therefore, porcine and feline/canine genotypes are of special interest with regard to their zoonotic potential, as they share 70% amino acid identity with human genotypes.

New variants, genotypes, and recombinants frequently emerge in the human population, yet their origin is unknown. It is assumed that these viruses emerge either from an unsampled population (e.g., asymptomatic or immunocompromised patients or demographic regions from which surveillance data are lacking) or from an animal reservoir. Anti-bovine and -canine norovirus antibodies have been reported in humans, and, conversely, various species of animals have tested positive for antibodies to human noroviruses [354]. Furthermore, viral RNA of human GI and GII strains has been found in fecal material of calves, pigs, birds, captive macaques, dogs, and rodents ([195, 199, 201, 208, 253, 256, 260-263, 266, 267]; reviewed in reference [354]). With the exception of birds and dogs (which have not been used for inoculation experiments), these species are also susceptible to human noroviruses under experimental conditions [51]. This implies that animals can be a reservoir for human noroviruses.

The best-studied susceptibility factors for human noroviruses are histo-blood group antigens (HBGAs) [67-69]. These terminal sugars of carbohydrate chains are linked to glycoproteins or glycolipids on red blood cells and tissues, including the epithelial cells of the gastrointestinal tract [70, 71]. Moreover, HBGAs are secreted by these cells into bodily fluids, including mucosa and saliva [72]. In the intestine, HBGAs are derived from precursor structures to which an α1,2-fucosyltransferase 2 (FUT2) adds a fucose group, resulting in the H1, H2, or H3 antigens. The addition of an α1,3 or α1,4-linked fucose group to the H1 or H2 antigen or their precursor structure results in the Lewis a, b, x, and y antigens. These steps are carried out by either the FUT3 to 7 or FUT9 enzyme. The A and B enzymes, encoded by the ABO locus, add either an *N*-acetylgalactosamine or a galactose in a α1,3 linkage to the H antigen, resulting in the A and B antigens, respectively [72].

Most human, canine, and bat noroviruses bind to synthetic HBGAs in a strain-dependent manner [170, 248, 351]. In contrast, bovine (GIII) and murine (GV) noroviruses recognize receptors that are not expressed in humans; GIII.2 (Newbury

agent 2) attaches to the alpha-galactosidase (Galα1,3), and the GV receptor is the transmembrane protein CD300lf, which is expressed on murine tuft cells in the intestine, but the main expression of this molecule is on hematopoietic cells [280, 282, 283, 367]. For other animal noroviruses, including the viruses identified in harbor porpoises (GNA1), sheep (GIII.2), and cats (GIV.2), no HBGA ligand or alternative attachment factors have been identified.

Norovirus attachment to HBGAs *in vitro* is assumed to be the primary step for virus uptake into the target cell. However, HBGA expression alone is not sufficient to enable infection of cells in culture. Therefore, it has been hypothesized that HBGAs are necessary for infection but not sufficient by themselves to initiate a full infectious cycle [368]. The important role that HBGAs play in norovirus susceptibility has been confirmed by volunteer studies and epidemiological data (reviewed in reference [77]). They indicated that a subset of the human population, which does not express HBGA in the mucosa or saliva, is resistant to certain norovirus strains. Dependent on geographical location, these non-secretors make up between 5% and 20% of the human population and do not express a functional FUT2, thereby lacking the H-antigen-based structures on their intestinal epithelium and in their saliva.

Binding studies using fluorescein isothiocyanate (FITC)-labeled viruses and formalinfixed paraffin-embedded (FFPE) tissues, or virus histochemistry, have been shown to be a valuable tool for studying host and cell tropism for viruses such as avian influenza virus and Middle East respiratory syndrome coronavirus (MERS-CoV) [369, 370], and we have now set up this technique for norovirus. The aim of the study was to assess the potential susceptibility of different animals to human noroviruses. As attachment is the first crucial step for a virus to initiate infection of a host cell, we tested attachment of a diverse range of human noroviruses using FITC-labeled virus-like particles (VLPs) on human (for validation) and animal FFPE tissues. The expression of the recombinant major capsid protein (VP1) results in self-assembly of empty capsids that are morphologically similar to the infective norovirus virions and therefore are commonly used as surrogates to study norovirus-HBGA interactions [371]. The FITC-label allowed us to study attachment of all genotypes by eliminating the need for secondary anti-norovirus antibodies that are currently only available for a limited selection of genotypes. To test if norovirus attachment is associated with a host HBGA profile, we defined the HBGA phenotype of these tissues using immunohistochemistry. Studying virus attachment to tissues from potential hosts will lead to a better understanding of which animal species are more likely to be susceptible to infection with human noroviruses and, therefore, focus efforts in the search for a reservoir for human noroviruses.

2. Results

2.1. FITC-labeled human norovirus VLPs attach to human intestinal epithelium

To validate binding of the FITC-labeled VLPs, we tested attachment of GII.4 Sydney 2012 on 6 human tissues, originating from duodenum, jejunum, or ileum. To exclude any effect of the FITC label on the VLP binding, we also tested the attachment of unlabeled GII.4 that was detected with an anti-GII.4 antibody. FITC-labeled and unlabeled GII.4 attached with similar efficiency to the epithelium of the villi and the crypts of tissues derived from duodenum, jejunum, and ileum of 3/6 human tissues (**Fig. 1A** and **C** and **Table 1**), while the negative control did not attach to any of the tissues (**Fig. 1B** and **D**). In one sample, additional staining was detected in the Brunner glands (**Fig. 1E** and **F**).

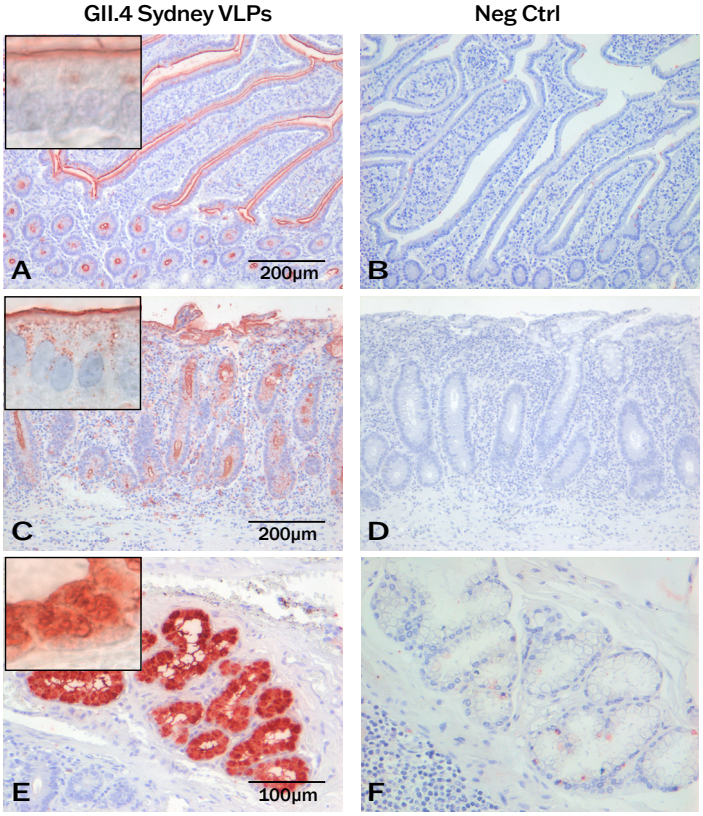

Figure 1. GII.4 VLPs attached to epithelium of villi and crypts in human small intestinal tissues (**red**). No difference was seen between (**A**) FITC-labeled and (**C**) unlabeled VLPs that were detected with an anti-FITC and an anti-GII.4 antibody, respectively. (**E**) In some tissues, VLPs additionally attached to the Brunner glands. (**B**, **D**, and **F**) No staining was seen in the negative controls (Neg Ctrl). Magnifications, 10× (**A**, **B**, **C**, and **D**) 20× (**E** and **F**). Picture insets of attachment signal are 100×.

GII.4 attachment depends on the α1,2-fucose group [372] that is added by FUT2, and we enzymatically cleaved this group with a 1,2α-fucosidase, which was confirmed by the *Ulex europeus* (UEA-I) lectin staining (**Fig. 2B**). Upon α1,2-fucose removal, GII.4 binding was completely lost (**Fig. 2C** and **D**). FITC-labeled and unlabeled GII.4 VLPs showed identical attachment patterns, indicating that FITC-labeled particles can be used to study attachment.

To further validate our assay and compare the attachment pattern of different human norovirus genotypes, the VLPs were first tested on human tissues. We included VLPs representing a wide range of human norovirus genotypes: GI (GI.3, GI.6), GII (GII.1, GII.3, GII.4, GII.6, and GII.17 (2005, 2014, and 2015), GIV.1, and GIX. All strains, except GII.1, attached to at least one of the human tissues. While all genotypes attached to the epithelium, variation was found in genotype binding between individuals, as not all strains attached to the same individuals (**Table 1**). Of all the human tissues, there was only one, a human ileum tissue, to which none of the VLPs attached. GI.6, GII.6, GII.17 (2014 and 2015), and GIX attached to 5/6 tissues, showing the broadest range of attachment. When we removed the α1,2-fucose group, thereby changing the HBGA to a nonsecretor phenotype, attachment of GII.3, GII.17, GII.6, and GIX was clearly reduced (**Fig. 2E** and **F**), while that of GI and GIV.1 was unaffected (**Fig. 2G** and **H**). Thus, VLPs from different genotypes attached to the epithelium of human tissue. The finding that not all VLPs attached to all tissues suggests the role of a host factor.

2.2. FITC-labeled VLPs of genogroups GI, GII, GIV, and GIX attach to animal intestinal tissue

To investigate the potential susceptibility of different animal species, we tested the attachment of the human norovirus VLPs on a variety of animal tissues. Tested were tissues of species or families in which human noroviruses have previously been detected or have been susceptible to human norovirus infection in the laboratory (**Table 1**). We further tested tissues of species that have developed and sustained their own noroviruses (**Table 1**) as well as oysters, which are known bioaccumulators of human noroviruses and in which a few animal noroviruses have been detected [294, 373].

Like on human tissue, GI, GII, GIV.1, and GIX VLPs also attached to intestinal epithelium of animals (**Fig. 3** and **Table 1**). Out of the 14 species tested, tissue samples from the black-headed gull and the straw-colored fruit bat were the only samples to which none of the VLPs attached. The highest diversity of genotype attachment was seen for the dog and the common pipistrelle bat samples, to which VLPs from all tested genotypes attached.

On the contrary, only a few genotypes attached to tissue samples from harbor porpoise (GI.3, GI.6, and GII.4), Egyptian fruit bat (GI.3, GI.6, and GIV.1), turkey (GI.3 and GI.6), and chicken (GI.3, GI.6, and GIV.1). The ability to attach to several species differed between VLPs of different genogroups and genotypes. For instance, GI.6 VLP attached

Table 1. Summary of the virus histochemistry and immunohistochemistry results.

Tissue source	Hitochemistry results[1]														HBGA
	GI.3	GI.6	GII.1	GII.3	GII.4	GII.6	GII.17 2005	GII.17 2014	GII.17 2015	GIV.1	GIX	GNA1	GIII.2 GII.18 GIV.2	Total	
Human[2]															
Human1 (ile)	-	-	-	-	-	-	-	-	-	-	-	-	-	0/15	A,B,Le^{a,x,y}
Human2 (jej)	-	+	-	+	+	+	-	+	+	+	+	-	-	8/15	A,H2,Le^{b}
Human3 (duo)	-	+	-	+	+	+	-	+	+	+	+	-	-	8/15	A,H2,Le^{b}
Human4 (ile)	+	+	-	+	+	+	+	+	+	-	+	-	-	9/15	H2,Le^{a,b,y}
Human5 (duo)	+	+	-	+	-	+	+	+	+	+	+	+	-	10/15	H2,Le^{a,b,x}
Human6 (duo)	+	+	-	-	-	+	+	+	+	+	+	+	-	9/15	Le^{a,b,x}
Animal[3]															
Pig1#$^{+}$	-	-	-	-	+	+	+	+	+	-	+	-	-	6/15	H1,H2
Pig2^{+}	-	+	-	-	-	-	-	+	+	+	+	-	-	5/15	A,H2
Pig3^{+}	-	-	-	-	-	-	-	-	-	-	-	-	-	0/15	H2
Pig4^{+}	-	+	-	-	-	-	-	-	-	+	-	-	-	2/15	A,H1,H2
Dog1^{+}	-	-	-	+	+	+	+	-	-	-	-	+	-	5/15	H1,H2
Dog2#$^{+}$	+	+	-	+	+	+	-	+	+	+	+	-	-	9/15	A,Le^{a,x,y}
Dog3^{+}	-	-	-	-	+	+	+	+	-	-	+	+	-	6/15	H1,H2
Cat1^{+}	+	+	-	-	+	-	-	-	-	-	+	+	-	5/15	A,H2,Le^{b,x,y}
Rat1^{+}	-	+	-	-	+	+	-	+	-	+	+	-	-	6/15	A,H1,H2
Rat2^{+}	-	-	-	-	+	-	-	-	-	-	+	-	-	2/15	H1,H2
Rat3^{+}	-	+	-	-	+	+	-	+	-	+	+	-	-	6/15	A,B,H1,H2
Chimpanzee1	-	+	-	+	+	+	-	+	+	+	+	-	-	8/15	A,H2,Le^{b}
Chimpanzee2	-	+	-	-	+	+	-	+	+	+	+	-	-	7/15	A,H2,Le^{b}
Porpoise1^{+}	+	+	-	-	+	-	-	-	-	-	-	+	-	4/15	H2,Le^{x,y}
Porpoise2^{+}	+	+	-	-	-	-	-	-	-	-	-	-	-	2/15	Le^{x,y}
Porpoise3^{+}	+	+	-	-	-	-	-	-	-	-	-	+	-	3/15	Le^{x,y}
Pipistrelle1^{+4}	+	+	-	-	+	+	+	+	-	-	+	+	-	8/15	A,H1,H2
Pipistrelle2^{+4}	+	+	-	-	+	+	-	-	-	+	+	-	-	6/15	A,B,H2,Le^{y}
Pipistrelle3^{+4}	+	+	-	-	+	+	-	+	+	+	+	-	-	8/15	A,B,H1,H2
Pipistrelle4^{+4}	+	+	-	+	+	+	+	+	+	+	+	+	-	11/15	A,H1,H2,Le^{x,y}
Straw-coloured FB1	-	-	-	-	-	-	-	-	-	-	-	-	-	0/15	B,Le^{a}
Straw-coloured FB2*	-	-	-	-	-	-	-	-	-	-	-	-	-	0/15	-
Straw-coloured FB3	-	-	-	-	-	-	-	-	-	-	-	-	-	0/15	B,Le^{y}
Egyptian FB1	+	+	-	-	-	-	-	-	-	+	-	-	-	3/15	B,Le^{a,x,y}
Egyptian FB2*	+	+	-	-	-	-	-	-	-	-	-	+	-	3/15	Le^{a,x}
Egyptian FB3	+	+	-	-	-	-	-	-	-	-	-	+	-	3/15	Le^{a,x,y}
Black-headed gull1#*[5]	-	-	-	-	-	-	-	-	-	-	-	-	-	0/15	-
Black-headed gull2*[5]	-	-	-	-	-	-	-	-	-	-	-	-	-	0/15	-
Black-headed gull3*[5]	-	-	-	-	-	-	-	-	-	-	-	-	-	0/15	-
Black-headed gull4*[5]	-	-	-	-	-	-	-	-	-	-	-	-	-	0/15	-
Mallard1*	+	+	-	-	-	-	-	-	-	+	-	+	-	4/15	Le^{x}
Mallard2#	+	+	-	+	-	+	-	+	-	+	+	-	-	7/15	Le^{a,b,x}
Mallard3	+	+	-	-	-	+	-	-	-	+	-	-	-	4/15	Le^{a,b,x}
Turkey1	+	+	-	-	-	-	-	-	-	-	-	-	-	2/15	Le^{b,x,y}
Chicken1	+	+	-	-	-	-	-	-	-	+	-	-	-	3/15	Le^{b,x,y}
Chicken2*	+	+	-	-	-	-	-	-	-	-	-	-	-	2/15	Le^{x}
Oyster1	-	-	-	-	+	-	-	-	-	-	-	-	-	1/15	A
Oyster2	-	+	-	-	+	-	-	+	-	-	+	-	-	4/15	A,H1
Oyster3	+	-	-	-	+	-	-	-	-	-	+	-	-	3/15	A
Oyster4	-	-	-	-	+	-	-	-	-	-	-	-	-	1/15	A,H1
Species positive/ Species tested[6]	10/15	13/15	0/15	5/15	9/15	7/15	4/15	8/15	5/15	9/15	9/15	7/15	0/15		
Individuals positive/ Individuals tested[6]	22/46	30/46	0/46	9/46	22/46	19/46	8/46	18/46	12/46	19/46	22/46	12/46	0/46		

[1] binding,+; no binding, -.; [2] duo=duodenum; jej=jejunum; ile=ileum; Le=Lewis; [3] Underlined are species in which human strains have been detected or which have been susceptible to human noroviruses in the laboratory; [4] GX was found in species belonging to another microbat genus, the horseshoe bat (*Rhinolophus*); [5] Human strains were found in faeces of gulls within the *Larus* genus; [6] Including humans; * Non-secretor animals; + species with own norovirus; # duodenum, jejunum and ileum were tested (**Table 2**).

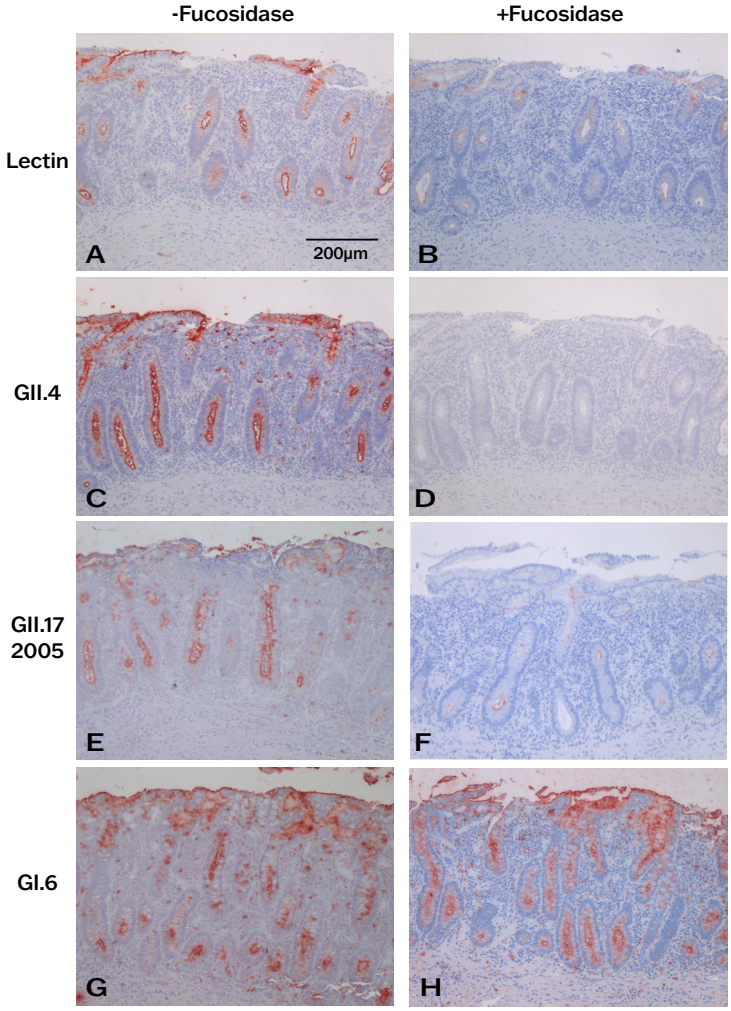

Figure 2. Attachment of VLPs to human intestinal tissue after enzymatic removal of the α1,2-fucose group. UEA-I lectin staining (**A**) without and (**B**) with fucosidase treatment was performed as a control to confirm cleavage of the α1,2-fucose group. Upon 1,2α-fucosidase treatment, no attachment of GII.4 was observed (**C** and **D**). Attachment of GII.3, GII.6, GII.17 (2005, 2014, and 2015), and GIX was reduced (represented by GII.17 2005 staining [**E** and **F**]). Attachment of GI.3, GI.6, and GIV.1 was unchanged (represented by GI.6 staining [**G** and **H**]). Magnifications, 10×.

to intestinal tissue from 13/15 species (including humans), whereas GII.17 (2005) VLP only attached to tissues from 4/15 species. Thus, although most genotypes were capable of attaching to animal tissues, we did observe genotype- and species-specific differences.

To test if attachment varies between different parts of the GI tract within one individual, we tested VLP attachment to duodenum, jejunum, and ileum for one pig, one dog, and

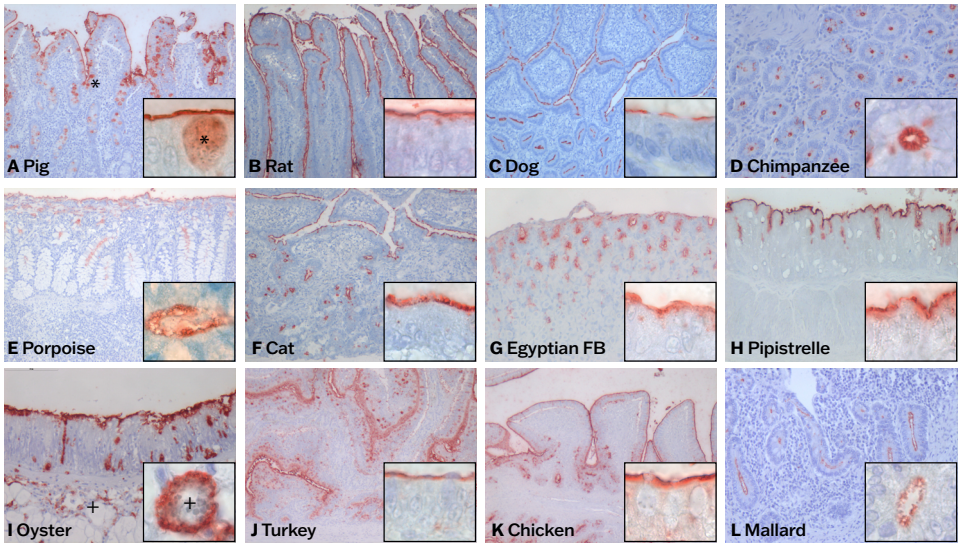

Figure 3. Human norovirus VLPs attached to the epithelium of intestinal tissues originating from various animals. A representation of different VLPs is shown here. In pigs and oysters, attachment was also detected intracellularly in goblet cells (*) and unidentified cells (+), respectively. Magnifications, 10× (A, B, C, E, F, J, and K), 20× (D, G, H, I, and L), and 100× (insets).

one mallard specimen for which these additional samples were available (**Table 2**). In the pig samples, VLPs attached only to the ileum and not the duodenum and jejunum. Conversely, in the dog, most genotypes attached to the ileum and jejunum and fewer to the duodenum. In the mallard, GII.17 (2014) and GIX VLPs attached exclusively to the jejunum and the cecum, GIV.1 VLPs to the duodenum, the jejunum, and the cecum, and GII.3 and GII.6 only to the cecum. GI.3 and GI.6 attached to all parts except the cecum. For some species, additional non-small-intestinal tissues were available, to which we noticed unexpected attachment, i.e., in the common pipistrelle (stomach, colon, and bladder), Egyptian fruit bat (colon), mallard (cecum), and cat (esophagus and stomach).

In conclusion, we detected the attachment of FITC-labeled VLPs of genogroups GI, GII, GIV, and GIX to intestinal epithelium of all animal species, except for the black-headed gull and the straw-colored fruit bat, and with the broadest range of VLPs attaching to dog and common pipistrelle tissues.

2.3. Porpoise norovirus attaches to animal and human tissues

In addition to human strains, we also tested animal norovirus genotypes, GII.18 (porcine), GIII.2 (bovine), GIV.2 (feline), and GNA1 (harbor porpoise), on human and animal tissues. For GII.18, we experienced problems expressing VP1, and we used

Table 2. Attachment of human noroviruses to different sections of the small intestine of one pig, one dog, and one mallard.

Animal[2]	Attachment in[1]													HBGA
	GI.3	GI.6	GII.1	GII.3	GII.4	GII.6	GII.17 2005	GII.17 2014	GII.17 2015	GIV.1	GIX	GNA1	GIII.2 GII.18 GIV.2	
Pig1 (duo)	-	-	-	-	-	-	-	-	-	-	-	-	-	H1,H2
Pig1 (jej)	-	-	-	-	-	-	-	-	-	-	-	-	-	H1,H2
Pig1 (ile)	-	-	-	-	+	+	+	+	+	-	+	-	-	H1,H2
Dog2 (duo)	+	+	-	-	-	-	-	-	-	+	+	-	-	A,$Le^{a,x,y}$
Dog2 (jej)	+	+	-	+	+	+	-	+	+	+	+	-	-	A,$Le^{a,x,y}$
Dog2 (ile)	+	+	-	+	+	+	-	+	+	+	+	-	-	A,$Le^{x,y}$
Mallard2 (duo)	+	+	-	-	-	-	-	-	-	+	-	-	-	$Le^{a,b}$
Mallard2 (jej)	+	+	-	-	-	-	-	+	-	+	+	-	-	$Le^{a,b}$
Mallard2 (ile)	+	+	-	-	-	-	-	-	-	-	-	-	-	$Le^{a,b}$
Mallard2 (cec)	-	-	-	+	-	+	-	+	-	+	+	-	-	$Le^{a,b,x}$

[1] binding,+; no binding, -.; [2] duo=duodenum; jej=jejunum; ile=ileum; cec=cecum, Le=Lewis.

P particles instead. The structural features and critical residues for HBGA binding are known for several GI and GII genotypes, while these are largely unknown for GIV.1 and some of the genogroups infecting animal species. Sequence alignment of all VLPs tested (**Fig. 4**) showed that there are large differences for the animal noroviruses compared to the known residues of the HBGA binding sites of GI and GII, indicating that these have different ligand binding sites.

The harbor porpoise strain GNA1 attached to epithelium of 2/3 porpoise specimens as well as to intestinal tissues of humans, dogs, cats, common pipistrelles, Egyptian fruit bat, and a mallard (**Table 1**). VLPs from none of the other animal strains attached to any of the tissues. Neither chemical (citric acid) nor enzymatic (protease) antigen retrieval treatment resulted in binding. For canine and bat noroviruses, an effect of temperature on binding has been reported [170, 248]. Therefore, we tested binding at various temperatures (4°C, 25°C, and 37°C) as well as increased VLP concentration, but we did not observe any binding. Thus, perhaps virus histochemistry is not a suitable method to study attachment for all animal norovirus strains.

2.4. Animals express HBGAs similar to humans

To determine whether VLP attachment can be associated with the expression of specific HBGAs, we characterized the HBGA profile of humans and potential animal hosts by immunohistochemistry (IHC) on the same FFPE tissues that had been used for virus histochemistry. Humans expressed H and Lewis antigens on the epithelium of the villi and crypts, and in some individuals additional expression was detected in the mucin-producing goblet cells and Brunner glands (**Tables 1** and **Table 3**). In

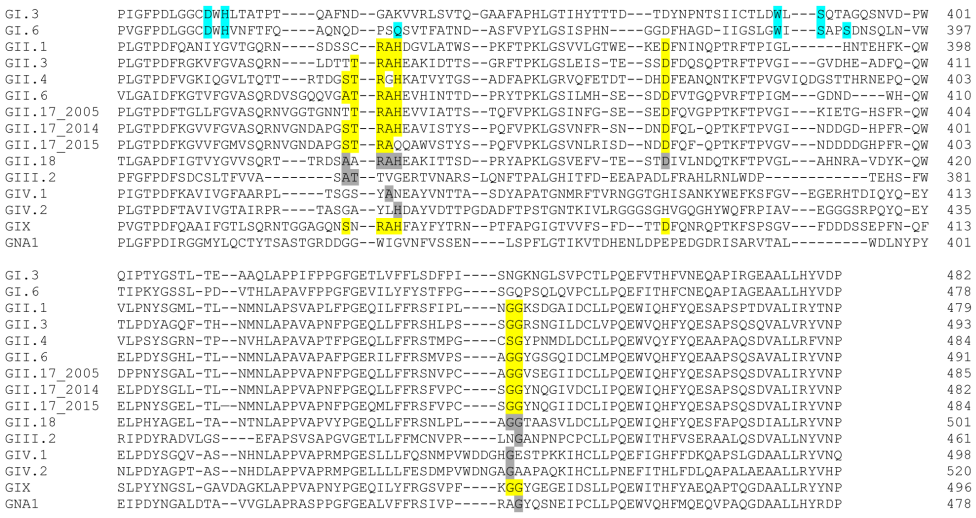

Figure 4. Sequence alignment of the HBGA-binding interfaces and the surrounding regions of human and animal norovirus genotypes used in this study. All VP1 sequences of VLPs (accession numbers are in **Table 4**) were aligned with MUSCLE. Amino acids that have experimentally been shown to be required for GII (**yellow**) and GI (**blue**) interaction with HBGAs are marked [21]. Amino acids that are identical to known HBGA interaction residues are shaded (**grey**). The number indicates the amino acid position counted from the start of VP1.

most animal tissues, HBGAs were expressed on the epithelium and sometimes in goblet cells and Brunner glands. Samples of humans, dogs, and the common pipistrelle expressed the widest diversity of HBGAs, being positive for H, A, and Lewis antigens. On the contrary, the bird species showed the lowest diversity, expressing only Lewis antigens. Tissues of the black-headed gull as well as one straw-colored fruit bat were the only tissues in which we did not detect any HBGA expression. It is known that the detection of the H1 and H2 antigens can be hindered by the presence of the A, B, Le^b, and Le^y antigens [170, 374, 375]. Therefore, secretors were defined as being positive for H1, H2, A, B, Le^b or Le^y. Of note, although defined as non-secretor antigens, Le^a and Le^x were also detected in secretors, indicative of FUT3 activity.

In total, only eight non-secretors were found, namely, within the chicken, the mallards, the black-headed gulls, the Egyptian fruit bats, and the straw-colored fruit bats (**Table 1**). Due to the limited number of non-secretor individuals, attachment data on these individuals were scarce, but GI.3, GI.6, GIV.1, and GNA1 were the only genotypes attaching to non-secretor tissues. For the tissue regions where VLPs attach to overlap with HBGA expression, we further observed differences in HBGA phenotype between species and between individuals. However, we did not find a one-to-one correlation between VLP attachment and specific ABH or Lewis phenotypes (**Table 1**). Similarly,

Table 3. Summary of HBGA expression in intestinal epithelium of humans and different animal species.

Host	Results for HBGA[1]									Summary per species	HBGA from prev studies	*FUT2* [284]
	A	B	Le^a	Le^b	Le^x	Le^y	H1	H2	Secretor			
Human	4/6	1/6	4/6	5/6	3/6	2/6	0/6	4/6	6/6	A, B, H2, $Le^{a,b,x,y}$	A, B, H1, H2, $Le^{a,b,x,y}$ [70, 71, 271, 375]	+
Pig	2/4	0/4	0/4	0/4	0/4	0/4	2/4	4/4	4/4	A, H1, H2	A, H1, Le^b [346]	+
Dog	1/3	0/3	1/3	0/3	1/3	1/3	2/3	2/3	3/3	A, H1, H2, $Le^{a,x,y}$	A, H, $Le^{a,b,y}$ [170, 389]	+
Cat	1/1	0/1	0/1	1/1	1/1	1/1	0/1	0/1	1/1	A, H2, $Le^{b,x,y}$	A, H	+
Rat	2/3	1/3	0/3	0/3	0/3	0/3	3/3	3/3	3/3	A, B, H1, H2	A, B, H [390-392]	+
Chimpanzee	2/2	0/2	0/2	2/2	0/2	0/2	0/2	2/2	2/2	A, H2, Le^b	A, H, lewis [393]	+
Porpoise	0/3	0/3	0/3	0/3	3/3	3/3	0/3	1/3	3/3	H2, $Le^{x,y}$	NA	+[2]
Pipistrelle	4/4	2/4	0/4	0/4	1/4	2/4	3/4	4/4	4/4	A, B, H1, H2, $Le^{x,y}$	NA	+
Straw-colored FB	0/3	2/3	1/3	0/3	0/3	1/3	0/3	0/3	2/3	B, $Le^{a,y}$	NA	-
Egyptian FB	0/3	1/3	2/3	0/3	2/3	2/3	0/3	0/3	2/3	B, $Le^{a,x,y}$	NA	-
Black-headed gull	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	-	NA	NA
Mallard	0/3	0/3	2/3	2/3	1/3	0/3	0/3	0/3	2/3	$Le^{a,b,x}$	NA	-
Turkey	0/1	0/1	0/1	1/1	1/1	1/1	0/1	0/1	1/1	$Le^{b,x,y}$	NA	-
Chicken	0/2	0/2	0/2	1/2	2/2	1/2	0/2	0/2	1/2	$Le^{b,x,y}$	NA	-
Oyster	4/4	0/4	0/4	0/4	0/4	0/4	2/4	0/4	4/4	A, H1	A, H1 [393, 395]	NA
Species positive/ Species tested	8/15	5/15	5/15	7/15	9/15	9/15	5/15	7/15	14/15			

[1] Number of positive individuals/individuals tested; [2] Dolphins; FB=fruitbat; NA=not available; Le=Lewis

differences in VLP binding in different sections of the intestine were not correlated with differences in HBGA expression (**Table 2**). We did, however, note that the dog and pipistrelle tissues to which the broadest diversity of VLP attached, were also the species where HBGA expression most closely resembled that in humans.

2.5. FITC-labeled VLPs attach to synthetic HBGAs.

To characterize specific VLP-HBGA interaction, we further investigated their binding to synthetic HBGAs. To exclude the interference of FITC, labeled and unlabeled GII.4 samples were tested for HBGA binding using either an anti-FITC or an anti-GII.4 antibody. GII.4 attached to H1, H3, A, B, Le^b, and Le^y independently of the FITC label, indicating that the FITC labeling does not impact the VLP-HBGA interaction (**Fig. 5**). We further tested the same panel of VLPs that had been used for virus histochemistry. As described by others, VLP-HBGA interaction varied between strains. GII.6 had the broadest binding pattern, attaching to A, B, H, and Lewis antigens (**Fig. 6A**). In contrast, GII.1, GII.3, GII.15, and GIV.1 VLPs did not attach to any of the tested HBGAs (data not shown). GI.3, GI.6, and GII.6 attached to both secretor (Le^b and Le^y) and non-secretor (Le^a and Le^x) antigens, while GII.4 and GII.17 2005 and 2015 exclusively bound to secretor antigens. The three GII.17 strains showed differences in their HBGA interactions. While all three bound strongest to H3 and Le^b, GII.17 2005

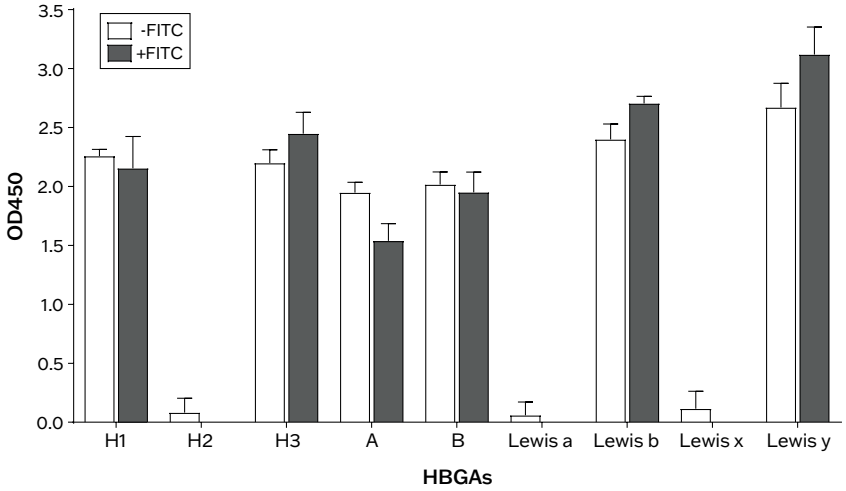

Figure 5. Binding of unlabeled (**white**) and labeled (**grey**) GII.4 VLPs to synthetic carbohydrates. Streptavidin plates were coated overnight at 4°C with 10 µg/ml PAA-conjugated HBGAs, and 100 ng of VLPs was incubated overnight at 4°C. Bound labeled and unlabeled VLPs were detected with an anti-FITC and an anti-GII.4 antibody, respectively. Each reaction was done in duplicates, and the error bars represent the standard deviations.

additionally bound to H1 and Le^y and GII.17 2014 to H1, H2, A, B, and Le^a. Of the animal VLPs, only the porpoise GNA1 attached to Le^x and Le^y, while the others did not bind to any of the HBGAs (**Fig. 6B**).

These data showed that GII.4 and GII.17 attached to secretor antigens only, while GI and GII.6 attached to secretor and non-secretor antigens. Interestingly, GII.3 and GIX, which contain the conserved residues within the HBGA binding site and attach to tissues, did not recognize any of the synthetic carbohydrates.

3. Discussion

This study demonstrates that a broad range of mammalian and bird species could be susceptible to human noroviruses based on their carbohydrate expression and attachment of human norovirus VLPs to intestinal tissue. Human norovirus VLPs attached to intestinal tissues that originated from species that have been found positive for human norovirus RNA, including pigs, dogs, rats, primates, and bird species. Dogs are the only animals for which human-to-animal transmission has been reported [253, 355]. Interestingly, we also detected broad attachment to species in which no human norovirus has been found to date. These include cats, the common pipistrelle, mallards, and, to a lesser extent, harbor porpoises, Egyptian fruit bats, turkeys, and chickens. Bats and birds are of special interest, as they are important reservoirs

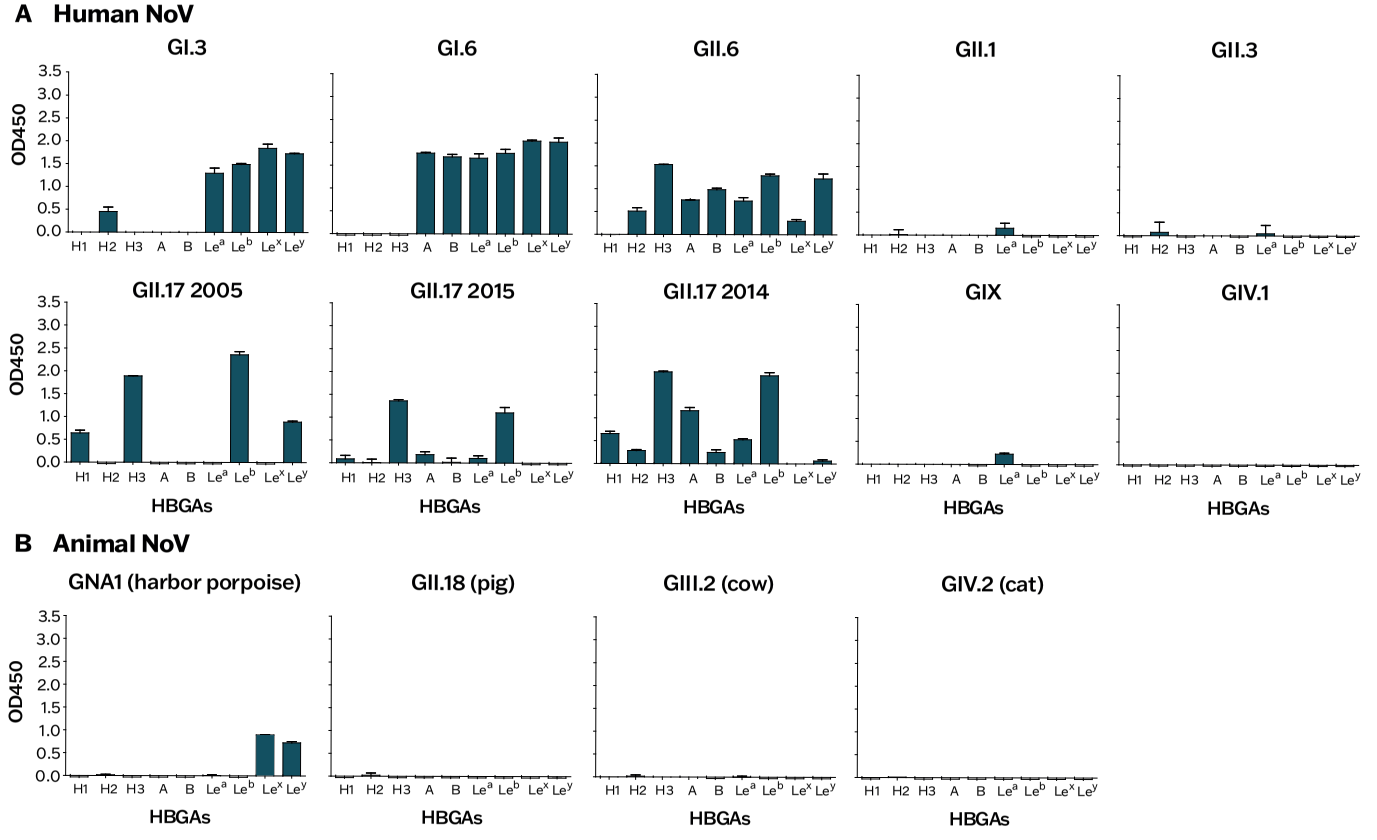

Figure 6. Binding of (**A**) human and (**B**) animal FITC-labeled VLPs to synthetic carbohydrates. Plates were coated with 10 µg/ml PAA-conjugated HBGAs. Attachment of 100 ng FITC-labeled VLPs was detected with anti-FITC antibody. Shown are the mean absorbance values, OD450, from which the negative-control values (D-mannose) have been subtracted. Each reaction was done in duplicates, and the error bars represent the standard deviations. Le=Lewis.

for many known human pathogens [285]. In bats, no human noroviruses have been found to date, although bat norovirus GX has been isolated from *Rhinolophus sinicus* and *Rhinolophus affinis* (also microbats) without obvious clinical signs. In wild birds in Finland and Brazil, RNA of human noroviruses (GII.4, GII.3, GI.2, and GII.p31) has been detected [266, 366]. The identified birds in Finland were mostly gull species from the genus *Larus*. The gulls that we tested, however, were from a different genus, *Chroicocephalus*, possibly explaining the lack of attachment of VLPs in this species.

In intestinal biopsy specimens of chronically infected patients, the dominant cell type found to be infected were enterocytes, which are the most prevalent cell type in the epithelium of the villi [47]. Similarly, in most animal species as well as the human samples, we detected VLPs that attach to the epithelium of villi. We also observed attachment to crypts, which have not been found to be positive in human biopsy specimens (42). *In vivo*, the crypts have a different environment than the villi, consisting of mostly nonenterocytes that secrete antimicrobial peptides and hormones, possibly hindering the ability of the virus to infect. VP1 antigen has also been detected in human biopsy specimens in macrophages in the lamina propria, and whole-virus staining with inactivated GII.4 Sakai showed binding exclusively to the lamina propria and Brunner glands [47, 376]. In contrast, we detected no attachment to immune cells in the lamina propria or Peyer's patches, supporting the hypothesis that staining in these cells could be due to phagocytosed infected enterocytes [47].

Depending on the animal model and genotype, viral antigen was previously detected in the duodenum, the jejunum, or the ileum without a tendency toward one location. Interestingly, we did see differences in attachment to different parts of the intestine in pigs, dogs, and mallards. For some species, additional non-small-intestinal tissues were available for which we noticed unexpected attachment. This indicates that there are differences between norovirus strains in their preference to infect certain parts of the intestine and that noroviruses, in some cases, are able to infect sites outside the small intestine. The latter has been observed in animal infection experiments with human norovirus strains where virus has also been detected in lymphatic organs [56, 57, 334].

There are intrahost and intra- and interspecies differences in the attachment of noroviruses. To investigate what underlies this, we compared VLP attachment to HBGA expression, which, in humans, is the best understood host susceptibility factor. Genetic analysis has shown that genes encoding enzymes that are involved in HBGA synthesis are found in a broad range of vertebrates and invertebrates [284]. As in humans, we found HBGAs expressed on the intestinal epithelium in a broad variety of animal species. Our findings of HBGA expression in the mallard, chicken, turkey, and megabat species is in contrast to the previous findings described by Yamamoto *et al.*, in which the genetic foundation of FUT and ABO genes across the animal kingdom was analyzed [284]. Megabats and bird species, including chickens, mallards, and

turkeys, were identified as lacking the ABO- and FUT-related genes. An explanation for this discrepancy could be that unrecognized homologs of the enzymes needed for the addition of the different antigens exist or, alternatively, that our antibodies are cross-reacting with similar carbohydrate structures.

VLP attachment did not correlate one-to-one with any specific ABH or Lewis phenotype, which has also been described in other studies [70, 271]. We did, however, notice that those specimens with a wider range of HBGA expression also showed attachment of more noroviruses. Dogs and the common pipistrelle expressed almost the same diversity of HBGA as humans, and, in accordance with this, all VLPs attached to tissues from these species. This indicates that dogs as well as some microbat species are susceptible to a broader range of noroviruses. The close contact between humans and dogs also makes them interesting candidates for human-to-animal transmission. Rather than specific HBGA expression, the secretor status might be more important for susceptibility, especially for GII genotypes. The dependence on the α1,2-fucose group is documented for GII.4 Sydney 2012 and GI.1 [70], and, accordingly, GII.4 only attached to secretor-positive animals. The limited number of eight non-secretor individuals does not allow us to draw conclusions, but GI and GIV.1 were the only VLPs attaching to these tissues. When treated with a 1,2α-fucosidase, GII.4 binding was completely diminished, while attachment of the other GIIs was reduced and that of GI and GIV.1 remained unchanged. This fits into the notion that attachment of GII strains depends on the α1,2-fucose group, while GI strains bind to a terminal Gal group [377]. Our data support the hypothesis that HBGAs are necessary but insufficient for norovirus attachment and infection. This was evidenced by the fact that HBGA expression alone did not automatically lead to VLP attachment and that, although within the same tissue VLP attachment was always co-located with HBGA expression, HBGA expression was also detected in locations where VLPs did not attach.

GII.4 is the genotype most frequently found in human outbreaks and the most commonly found human genotype in animals [31, 354]. Therefore, it is surprising that not GII.4 but GI.3 and GI.6 were the strains that attached to the broadest range of species (10 and 13/15 species, respectively). Similarly, the broad attachment of GIV.1 and GIX was unexpected, as the GIX genogroup consists of only a few reported strains, and GIV.1 strains, although found in sewage, are only sporadically detected in humans [145]. On the one hand, this indicates that attachment alone is not an indicator of how likely a strain is causing symptomatic outbreaks. On the other hand, attachment can also be affected by differences in stability. For example, in oysters, GI.1 has been shown to bioaccumulate more efficiently than GII.4, because the latter had been degraded in salt water [378]. We detected attachment of GII.4 to all oysters, which would likely be impacted by the conditions in salt water.

We observed a lack of binding of some human and most animal strains to synthetic glycans. We did not detect attachment the human GII.1, GII.3, GIV.1, and GIX strains to synthetic carbohydrates, even though HBGA ligands have been described and included for all but GIV.1 [21]. GNA1 was the only animal strain that attached to intestinal tissue as well as to synthetic glycans, Le^x and Le^y, which were also the only glycans expressed on harbor porpoise tissue. Some of the other animal genotypes have been tested before, and the lack of attachment was expected. These include GIV.2 and GII.11 and GII.19, which did not attach to HBGAs or human saliva, respectively [211, 350, 379]. Interestingly, the porcine genotypes have the conserved binding site, but no attachment factor has been identified yet. The change of two amino acids adjacent to GII.11 and GII.19 HBGA binding led to their attachment to type A and B antigens [379]. Similarly, GIII.2 attaches to α-galactosidase, which was not included in our glycan panel [280]. These variations in VLP-HBGA interaction data could be a result of several factors. They include the nature of the carbohydrates themselves (i.e., linker, mono- versus polysaccharides, and manufacturer) as well as the condition under which the interaction is tested (i.e., pH, temperature, and salt content). Differences in binding specificity have been shown for different strains of the same genotype. The labeling of the particles with FITC occurs at pH 9.6, although subsequent steps are performed at pH 7.5. Noroviruses and VLPs are stable at a pH range of 3 to 7 [380]; however, conformational changes have been reported, which could have implications for HBGA binding. Therefore, the lack of binding of some human as well as animal genotypes could be a result of the restricted selection of glycans that we tested as well as variation in stabilities of genotypes at high pH. For some genotypes and variants, no HBGA binding partner has been identified to date (including some GII.1 variants), and alternative or additional attachment molecules have been proposed, such as heparan sulfate [277, 278] and sialic acids [275]. Further, it cannot be excluded that a protein receptor plays a role for some strains, similar to what is observed for murine norovirus.

In summary, using FITC-labeled VLPs is a promising method to investigate potential tissue and cell tropism of different genotypes and strains, as it eliminates the need for specific antibodies. We have shown that many animals express HBGAs on their intestinal tissues, and additional factors are likely important for norovirus attachment and host susceptibility. Nevertheless, this is a first approximation to identify potential norovirus hosts and reservoirs. The broad attachment of many VLPs to these tissues should be followed up by investigating whether attachment subsequently leads to infection and if these are isolated or frequently occurring transmissions.

4. Materials and Methods

4.1. Plasmid constructs

VP1 sequences of human and animal noroviruses were custom synthesized (Idt) and cloned into pCAGGS with EcoRI and XhoI restriction sites added to the 5' and 3' ends, respectively. VP1s of GIV.1, GII.4, and GII.17 2015 were amplified from stool samples, and sequences were submitted to GenBank (accession numbers are listed in **Table 4**). RNA was isolated with the high-pure RNA isolation kit (Roche) and 5 µl of RNA, 1 µl (2 pmol) of random primers, 1 µl deoxynucleotide triphosphates (dNTPs; 10 mM each), 0.5 µl (20 U) RNase inhibitor. Samples were incubated for 5 min at 65°C and cooled down on ice for 5 min. 1× SuperScript IV RT buffer, 1 µl 0.1 M dithiothreitol, 0.5 µl (20 U) RNase inhibitor, and 1 µl (200 U) SuperScript IV RT buffer were added and incubated for 5 min at 25°C, 10 min at 50°C and 10 min at 80°C. Five microliters of the resulting cDNA was used for specific VP1 amplification with 1.25 µl dNTPs, 1× Pfu buffer, 1 µl Pfu, and 10 pmol primers containing EcoRI and XhoI restriction sites (underlined): GIV.1, AAAGAATTCATGAAGATGGCGTCGAGTGA/TAGCTCGAGTTATTGAAACCTCACTCTAC; GII.4, GGAGAATTCTGAAGATGGCGTCGAGTGAC/GTTCTCGAGTTATAGTGCACGTCTACGCCCCGTTC; GII.17 2015, GGAgaattcATGAAGATGGCGTCGAATGAC/GTTctcgagTTACTGAGCCCTCCTTCGCCCATT. The PCR protocol consisted of 2 min at 95°C and then 39 cycles of 30 s at 95°C, 1 min at 55°C, and 2.5 min at 72°C, followed by an elongation step of 6 min at 72°C. The PCR product was purified and ligated into the pCAGGs plasmid. The accuracy of the plasmids was checked by Sanger sequencing.

Table 4. VP1 sequences used to produce VLPs.

Genotype (ORF2)	Accession Nr
Human Norovirus	
GI.3	JQ911594
GI.6	LN854564
GII.1	LN854570
GII.3	LN854569
GII.4 (Sydney 2012)	MT232050
GII.6	KJ407072
GII.17 2005	DQ438972
GII.17 2014 (Kawasaki323)	AB983218
GII.17 2015	KX424646
GIX (prev GII.15)	KJ196290
GIV.1	MT232232
Animal Norovirus	
GNA1 (Porpoise)	KP987888
GIV.2 (cat)	JF781268
GIII.2 (cow)	AF320625
GII.18 (pig)	AY823305

4.2. VLP production and FITC labeling

VLPs were produced by adapting a previously published protocol [62, 381]. Twenty-four hours prior to transfection, 3 × 10^6 293T cells were seeded in gelatinized 10-cm plates in Dulbecco's modified Eagle's medium (DMEM; Lonza) supplemented with 10% fetal bovine serum (Sigma-Aldrich), 1× nonessential amino acids (Lonza), 1× PenStrep (Lonza), 1× L-glutamine (Lonza), and 1× sodium pyruvate (Gibco). Cells were kept at 37°C with 5% CO_2. The pCAGGs-VP1 construct was transfected using

calcium phosphate transfection. As a negative control, the pCAGGS construct without insert was transfected. For transfection, 6.2 µl $CaCl_2$, 40 µl plasmid, and 400 µl sterile water were mixed, and 500 µl HEPES buffered saline solution (8.18% NaCl, 5.94% HEPES, and 0.2% Na_2HPO_4 [all wt/vol]) was added. After 5 min, the transfection mix was added to the cells and incubated at 37°C for 16 h. Cells were then washed with phosphate-buffered saline (PBS) and the medium was refreshed. After another 48 h, the cells were harvested and centrifuged at 1000 rpm for 10 min. The pellet was resolved in lysis buffer (10% Triton X-100 with protease inhibitor [cOmplete Mini EDTA-free protease inhibitor cocktail; Roche]) and left for 5 min at room temperature. All subsequent centrifugation steps were done at 4°C. The lysate and supernatant were cleared by centrifugation at 3000 rpm for 15 min. The supernatant was then centrifuged through a 20% (wt/wt) sucrose cushion for 2 h at 27,000 rpm (SW32 rotor). The pellet was dissolved in 1 ml PBS for 20 min at 4°C and subsequently centrifuged overnight through a 20% to 60% sucrose gradient at 30'000 rpm (SW41 rotor). Fractions corresponding to 35% to 45% (w/w) sucrose were concentrated and washed through a 100-kDa Amicon filter at 4000 × g for 20 min at 4°C. The presence of VP1 was confirmed by SDS-PAGE. For GII.18, we experienced problems expressing VP1; therefore, we produced P particles as described before [382]. The presence of VLPs and P particles was confirmed by electron microscopy. VLPs in PBS were stored at -80°C until further used. VLPs and P particles were FITC-labeled as previously described [369]. Equal amounts of VLPs and 0.1 mg/ml FITC (Sigma-Aldrich) in 0.5 mol/liter bicarbonate buffer (pH 9.5) were mixed under constant stirring in the dark for 1 h. To lose excessive unbound FITC, the samples were dialyzed against PBS overnight in dialysis cassettes (GeBaFlex-Midi tubes, 8-kDa cutoff). VLPs were aliquoted and stored at -80°C until used. For quantification, FITC-labeled VLPs were run on a 12.5% acrylamide gel together with a bovine serum albumin (BSA) concentration marker. Silver staining was done with the silver staining kit (Thermo Fisher) according to the manufacturer's instructions.

4.3. Intestinal tissues

Human intestinal tissues were obtained from the Pathology Research and Trial Service (PARTS) at Erasmus MC. The following archival FFPE sections were obtained from the Department of Virology, Erasmus MC: harbor porpoises (*Phocoena phocoena*, n=3) that had been part of an unrelated study [383], chimpanzees (*Pan troglodytes*, n=2), domestic cat (*Felis catus,* n=1), which had been the negative-control animal in an unrelated animal study [384], chicken (*Gallus gallus domesticus*, n=2), which were the negative-control animals in an unrelated study [385], wild mallards (*Anas platyrhynchos*, n=3), black-headed gulls (*Chroicocephalus ridibundus,* n=4) that were from experiments with wild animals that had been published previously [386], common pipistrelles (*Pipistrellus pipistrellus,* microbat, n=4), Egyptian fruit bats (*Rousettus aegyptiacus,*

megabat, n=3), and straw-colored fruit bats (*Eidolon Helvum*, megabat, n=3). The bat samples were used in an unrelated study [387]. The rats (*Rattus norvegicus* n=3) were published in an unrelated study [388]. The pacific oysters (*Crassostrea gigas*, n=4) were collected from a market. Domestic pigs (*Sus scrofa domesticus,* n=4), dogs (*Canis lupus familiaris,* n=3), and turkey (*Meleagris gallopavo*, n=1) had been used for unrelated purposes. If available, we included three healthy individuals per species.

4.4. HBGA typing by immunohistochemistry

FFPE tissues were obtained from the Department of Pathology at Viroscience Erasmus MC. Three micrometer FFPE tissue slides were deparaffinized with xylene and hydrated using a graded ethanol series (100>100>95>90>70%). HBGA expression was assessed by immunohistochemistry (IHC). To block endogenous peroxidase, slides were incubated with 3% H_2O_2 diluted in PBS at room temperature (RT) for 10 min. All antibody incubation steps were conducted in 0.1% BSA for 1 h at RT with two washing steps (PBS plus 0.01% Tween) in between. HBGAs were detected with primary antibodies against antigen A (1:1; 9113D10; Diagast), B (1:1; 9621A8; Diagast), AB (1:1; 9113D10+152D12; Diagast), Neg (Diagast), Le^y (1:50; H18A; Absolute Antibody), Le^a (1:50; 7-LE; Sigma-Aldrich), Le^b (1:50; 2-25LE; Sigma-Aldrich), Le^x (1:100; MC480; Thermo Fisher), and H1 (1:100; 17-206; Thermo Fisher). A secondary biotinylated rabbit anti-mouse (1:100; Dako) was used, followed by Streptavidin-horseradish peroxidase (HRP)-conjugated antibody (1:300; Dako). Peroxidase was revealed with 3-amino-9-ethyl-carbazole (Sigma-Aldrich). Tissues were counterstained with hematoxylin and embedded in Meyer's glycerol-gelatin (Merck).

H type 2 was detected by lectin staining. Slides were blocked with 1% BSA in Tris-buffered saline (TBS; 50 mM Tris-HCl pH 7.5), and biotin-labeled *Ulex europeus* lectin (1:200 UEA-I; Sigma-Aldrich) was added in TBS with 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM $CaCl_2$, pH 7.5, and incubated overnight at 4°C. Slides were washed with TBS, and Streptavidin-HRP diluted in 1% BSA in TBS was added for 1 h at RT. The rest was done as described above.

4.5. Virus histochemistry on tissue sections

Tissue slides were prepared as for IHC (described above). For all blocking and antibody steps, TNB (0.1 M Tris, 0.15 M NaCl, pH 7.5, with 0.5% blocking reagent; Perkin Elmer) was used. Slides were blocked for 30 min at RT, and 25 ng of VLP or negative control was added and incubated overnight at 4°C. Between all subsequent steps, slides were washed twice with 0.01% Tween 20 in PBS, and all incubation steps were done at RT. Virus was detected by peroxidase-labeled rabbit anti-FITC (1:100; Dako) for 1 h. The signal was amplified using a tyramide signal amplification system (Perkin Elmer, Boston, MA) according to the manufacturer's instructions. Streptavidin-HRP was

added at 1:300 and incubated for 30 min. The HRP revelation was done as described above for IHC. As a control, unlabeled VLPs were used and stained with a primary anti-GII.4 antibody (1:100; SMV59; Maine Biotechnology, Portland ME, USA). The rest of the steps were done as described above for the IHC. The treatment with the 1,2α-fucosidase (kindly provided by Takane Katayama, Kyoto Unversity, Japan) was done prior to incubation with VLPs or the lectin staining. Twenty micrograms were added per slide in 100 mM sodium phosphate buffer, pH 6.5, overnight at 37°C.

4.6. Enzyme-linked immunosorbent assay (ELISA)-based carbohydrate microtiter plate assays

Streptavidin-coated high-capacity plates (Pierce, Invitrogen) were coated overnight at 4°C with 10 μg/ml biotinylated synthetic oligosaccharides that were polyacrylamide (PAA)-conjugated and that were eluted in 1× TBS buffer (20 mM Tris, 150 mM NaCl, pH 7.2). The carbohydrates were ordered at GlycoTech, Carbosynth, and GlycoNZ and are listed in **Table 5**.

Table 5. Synthetic HBGA structures used for the binding assay.

Name	Structure	Category	Manufacturer
Lewis d (H type 1)-PAA-biotin	Fucα1-2Galβ1-3GlcNAcβ1	Trisaccharide	Carbosynth
H (type 2)-PAA-biotin	Fuca1-2Galβ1-4GlcNAcβ1	Trisaccharide	Glycotech
H (type 3)-PAA-biotin	Fucα1-2Galβ1-3GalNAcα1	Disaccharide	Glycotech
Blood type A (tri)-PAA biotin	GalNAcα1-3(Fucα1-2)Galβ1	Trisaccharide	Glycotech
Blood type B (tri)-PAA biotin	Galα1-3(Fucα1-2)Galβ1	Trisaccharide	Glycotech
Lewis a-PAA-biotin	Galβ1-3(Fucα1-4)GlcNAcβ	Trisaccharide	Glycotech
Lewis b-PAA-biotin	Fucα1-2Galβ1-3(Fucα1-4)GlcNAcβ	Tetrasaccharide	Glycotech
Lewis x-PAA-biotin	Galβ1-4(Fucα1-3)GlcNAcβ1	Trisaccharide	Glycotech
Lewis y-PAA-biotin	Fucα1-2Galβ1-4(Fucα1-3)GlcNAcβ1	Tetrasaccharide	Glycotech
α-D-mannose-PAA-biotin	α-D-Man		GlycoNZ

Glc=glucose; Fuc=fucose; Gal=galactose; GlcNac=N-acetylglucosamine; Lac=lactose; GalNac=N-acetylgalactosamine; Man=Mannose

Plates were washed five times with cold PBS and blocked at RT with 5% BSA in PBS. After blocking and between all subsequent incubation steps, plates were washed five times with 0.01% Tween 20 in PBS (PBS-T). One hundred nanograms of FITC-labeled or unlabeled VLPs in 0.1% BSA in PBS-T was added and incubated overnight at 4°C. FITC-labeled VLPs were detected with an HRP-conjugated anti-FITC antibody (1:1000; Dako) for 1 h at 4°C. The unlabeled VLPs were detected with a mouse anti-GII.4 (1:100; SMV59; Maine Biotechnology, Portland, ME, USA), followed by an anti-mouse-HRP antibody (1:100; Dako).

The peroxidase signal was detected using the TMB 2-component microwell peroxidase substrate kit (SeraCare). The reaction was stopped after 10 min with 3 M H_2SO_4, and the mean absorbance values, i.e., the optical densities at 450 nm (OD_{450}), were measured, followed by subtraction of the negative control values (D-mannose). Each VLP was tested in duplicates.

Data availability: Sequences determined in the course of this work were submitted to GenBank (accession numbers are listed in **Table 4**).

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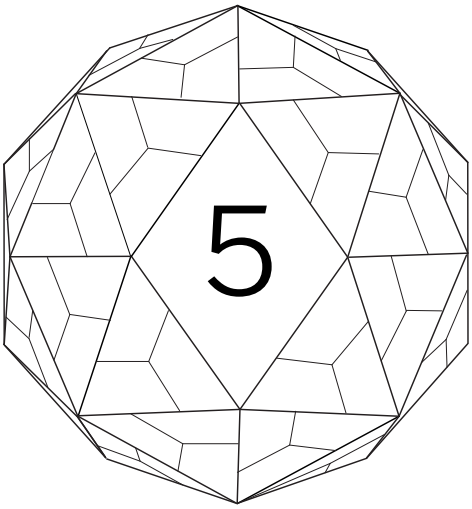
Conflicts of Interest: The authors declare no conflict of interest.



Capturing norovirus transmission

Miranda de Graaf, Nele Villabruna and Marion P.G. Koopmans

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Abstract: Human norovirus is a leading cause of gastroenteritis and is efficiently transmitted between humans and around the globe. The burden of norovirus infections in the global community and in health-care settings warrant the availability of outbreak prevention strategies and control measures that are tailored to the pathogen, outbreak setting and population at risk. A better understanding of viral and host determinants of transmission would aid in developing and fine-tuning such efforts. Here, we describe mechanisms of transmission, available model systems for studying norovirus transmission and their strengths and weaknesses as well as future research strategies.

Introduction

Human norovirus (HuNV) has been described as the perfect human pathogen due to its ability to replicate to high titers, its low infectious dose and high stability in the environment [517]. HuNV belongs to the family Caliciviridae and is the most prevalent viral cause of gastroenteritis cases and outbreaks worldwide, leading to significant morbidity and mortality [66, 518, 519]. To be maintained in the human population, it has to overcome environmental barriers as well as structural, functional and immunological barriers within the host and undergo a full replication cycle leading to the formation and release of new infectious virus particles. Next, these newly formed virus particles have to result in infection of additional cells within the same host and/or shedding from the host. Finally, the viral particles need to be transmitted to a new host, which occurs through the fecal-oral or oral-oral route. Transmission can be direct (person-to-person) or indirect through fecal or vomit contamination of food, water, fomites and the environment [27-30, 520].

The norovirus (NV) genus can be subdivided in seven genogroups, of which genogroups GI, GII and GIV have been detected in humans, and can be further subdivided into more than 40 genotypes [172]. These genotypes are not equally prevalent as causes of disease in humans: currently most gastroenteritis outbreaks are caused by the GII.4 genotype, although in some parts of Asia GII.17 recently emerged as the predominant genotype [259, 521, 522]. While the dynamics of GII.4 circulation are thought to be influenced by virus evolution and population immunity, it is not clear why this particular genotype is more successful than others in causing outbreaks and spreading around the globe. This is partly because much of our knowledge on NV transmission is based on epidemiological observations, rather than on controlled *in vitro* or *in vivo* experiments. Similarly, many other questions have remained unanswered. For example, what is the effect of antigenic evolution or recombination on norovirus fitness and transmissibility. What is the size and nature of genetic bottlenecks during transmission events? And what proportion of viruses that we can detect in a clinical or environmental setting are actually infectious and able to transmit? As major advances have been made in recent years, we review the currently available tools and models to study norovirus transmission *in vitro* and *in vivo*.

Evidence from epidemiological studies and outbreak investigations

Epidemiological studies have demonstrated that the contributions of the individual genogroups and genotypes can vary among outbreak settings and transmission routes (**Fig. 1**) [6]. For example, norovirus outbreaks caused by the GII.4 genotype are more common in health-care facilities than outbreaks caused by GI and non-GII.4 genotypes. Within health-care facilities GII.4 strains are more often associated with outbreaks in adult wards and GII.3 strains with outbreaks in children wards.

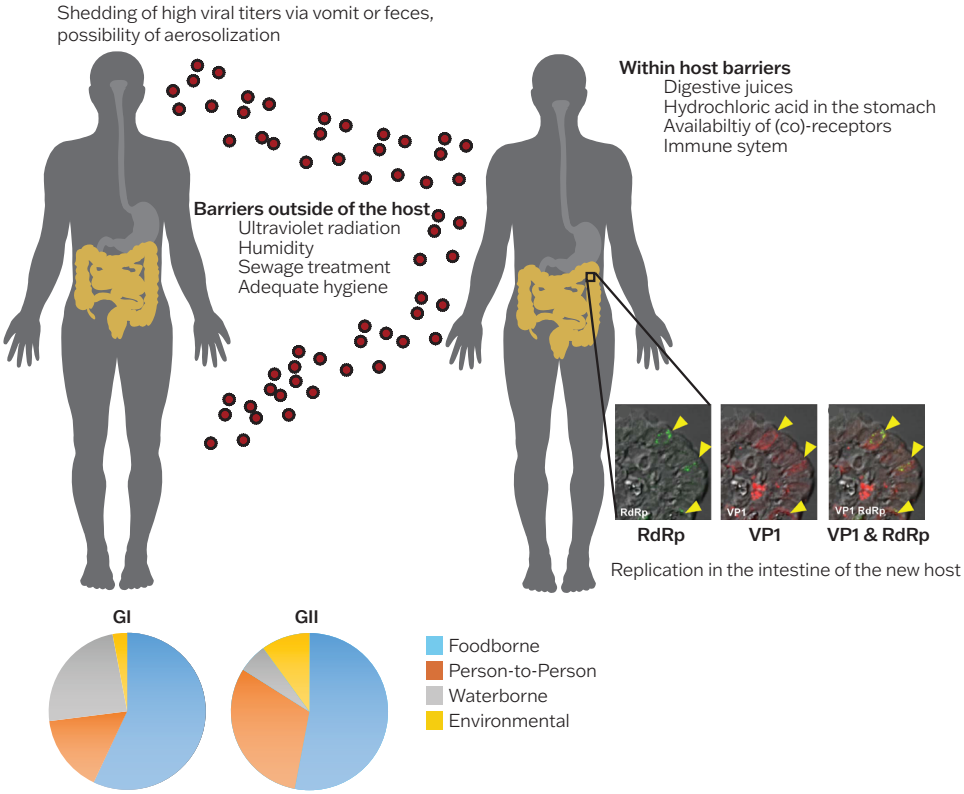

Figure 1. HuNV tropism and transmission. After shedding from the host via vomitus or feces HuNV is transmitted to the next host. Transmission can occur via several routes, with differences in association between genogroup and transmission route [552]. During transmission, viruses encounter multiple environmental and within host barriers that can potentially restrict or prevent transmission. After infection of the new host HuNV replicates in the intestine. In immunocompromised patients HuNV antigens can be detected in the ileum, jejunum and duodenum in enterocytes, macrophages, T-cells and dendritic cells [47]. The inset shows the detection of RdRp and VP1 (**yellow arrows**) in the same duodenal biopsy from a HuNV positive patient (adapted from [47]).

In hospitals the dominant transmission route is from patient-to-patient followed by patient-to-health-care worker and is related to level of dependency [523, 524]. In community outbreaks young children (<5 years) are more likely to infect other people compared to older children, possibly because they have relatively high rates of contact and low levels of hygiene [43, 44, 525]. Persons can be infected with HuNV without the presentation of symptoms [130], but the relationship between shedding and disease is not clear with some conflicting evidence in literature [126, 519].

However, in health-care settings, symptomatic patients were found to be responsible for the majority of transmission events [122]. In all, epidemiological studies have also provided some information on differences in transmission efficiency between genotypes, but it is difficult to obtain conclusive evidence without the use of *in vitro* and *in vivo* (transmission) model systems.

In vitro cell culture systems

Historically, norovirus transmission studies have been hindered by the lack of cell culture models. Noroviruses attach to human cells through the (co)-receptor histo-blood group antigens (HBGA) and a recent study demonstrated that HuNV productively infects B cells, in the presence of exogenous HBGA or HBGA-like molecules on specific intestinal bacteria [65, 526]. In biopsies obtained from HuNV infected immunocompromised persons, the major capsid protein (VP1) was detected in enterocytes, macrophages, T-cells and dendritic cells. HuNV replication was investigated by the detecting of the non-structural proteins RNA-dependent RNA polymerase (RdRp) and the genome associated VPg. Both were detected alongside with VP1 in duodenal and jejunal enterocytes (**Fig. 1**) [47]. In agreement with these findings, successful cultivation of multiple HuNV strains in human intestinal enteroid monolayers was recently reported [63]. Bile was required for replication of some strains, while the lack of appropriate HBGA restricted replication. *Ex vivo* inoculation of human duodenal tissues with GII.4 isolates also resulted in an increase of viral genomic RNA over time and expression of both structural and non-structural proteins in glandular epithelial cells [527]. These HuNV cell culture models can be used study HuNV replication kinetics, virus-host interactions and other aspects of NV biology and will finally allow researchers to address many of the unanswered questions listed above.

But what are the minimal requirements for a cell culture system to be a valuable tool for HuNV transmission studies? The HuNV cell culture system has to support attachment, internalization, replication and release of the viral particles, but is it necessary to include the microbiome? To study the role of bacteria, transwell cultures can be used where the viruses and bacteria are added to the apical or basolateral sides of the cell culture [528]. However, these infection models are not suitable for coculturing with a living

microbiome for prolonged periods of time, because of rapid bacterial overgrowth, which is a major limitation to their use. In the future, the gut-on-chip system could potentially mimic the normal epithelial differentiation in the gut ecosystem, in which peristalsis and flow of intestinal content restrain microbial overgrowth *in vivo* [529].

Experimental transmission models

HuNV infection and transmission events can be studied *in vivo* by the use of human volunteers, experimental animals or animal caliciviruses in their natural hosts. Several experimental animal models support HuNV replication; chimpanzees, immunocompromised mice, gnotobiotic pigs and gnotobiotic calves [55, 57, 58, 337]. Most of these animal models can be infected by HuNV via oral inoculation. Despite the apparent stability of NV in an acidic environment [530], for most of these studies sodium bicarbonate is orally administered prior to virus inoculation to neutralize stomach acids and increase infection efficiency. The immunocompromised BALB/c Rag-γc mice are an exception as they require an intraperitoneal route of infection, which is not ideal for transmission studies [58].

Pigs are natural hosts for norovirus genotypes GII.11, GII.18 and GII.19 [172, 190] while bovine species are natural hosts for GIII strains [345]. One study reports the detection of GII.4 in pigs and cattle from farms [195] and inoculation of both gnotobiotic pigs and calves with GII.4 results in replication [55]. However, these animal models are challenging due to size of the animals and costs. Replication in the gnotobiotic pig model occurs in the small intestine and results in virus shedding and diarrhea [53]. Contaminated oysters can be a source of foodborne HuNV infection in human and this can be mimicked in gnotobiotic pigs as they can be infected by feeding them HuNV seeded oyster homogenates [327]. In immunocompromised patients, HuNV infection can result in prolonged shedding and more severe disease, raising questions about the role of such persons in the emergence and transmission of HuNV [531]. Recently an immunocompromised gnotobiotic pig model was developed; these RAG2/IL2RG deficient pigs were characterized by depletion of lymphocytes and either absence of or structurally abnormal immune organs [330]. Similar to what was observed for immunocompromised patients, infection with GII.4 led to increased viral titers and prolonged virus shedding compared to wild-type pigs. An intriguing observation was that the use of a common treatment with cholesterol lowering drugs affected severity in humans, and HuNV replication in pigs [532, 533]. These results suggest that the gnotobiotic pig model is a suitable model to study foodborne transmission and transmission events involving immunocompromised patients. Co-infections with HuNV and HBGA-expressing *Enterobacter cloacae* were also investigated in the gnotobiotic pig model, but surprisingly and in contrast to the *in vitro* observations [534], co-inoculation with *Enterobacter cloacae* inhibited HuNV infectivity in pigs [333].

Chimpanzees can be infected with GI.1 HuNV by the intravenous and oral route [57, 337]. Infection does not result in diarrhea or histopathological changes of the gut tissue, although the duration and titers of HuNV shedding in feces resembles that in humans [57]. The virus could be passaged from chimpanzee-to-chimpanzee by feeding of fecal filtrate [337]. Notably, chimpanzees that were not challenged but were located in the same and adjacent rooms developed antibody responses, although HuNV antigen could not be detected in their feces [337]. However, chimpanzees are no longer available for biomedical research due to ethical reasons. Human GII.4 strains also have been detected in dogs and, surprisingly, canine seroprevalence to different HuNV genotypes resembles the seroprevalence in the human population [253, 255]. However, to date experimental infections of dogs with HuNV have not been documented.

The murine norovirus (MNV) model has been used to study many aspects of the NV replication cycle [12]. MNV belongs to genogroup GV and replicates to high titers *in vitro* and *in vivo* in its natural host [12, 535]. MNV and other cultivable caliciviruses such as Tulane virus (genus *Recovirus*) and feline calicivirus (genus *Vesivirus*) have been used as HuNV surrogates for inactivation studies, to either prevent transmission and control outbreaks or to increase food safety [536]. However, the value of these model organisms needs to be assessed on a case-by-case basis, depending on the question addressed as there can be differences in, amongst others, receptor usage and transmission routes [536]. These studies can also be performed with HuNV; the degradation of virus particles can be assessed by determining the change in viral RNA copies or binding properties [537], but such assays do not always accurately represent infectious titers.

A transmission model for MNV was developed by Rocha-Pereira *et al*. (**Fig. 2**) [538]. Donor animals were inoculated with MNV and placed into the same cage as uninfected sentinel animals. Alternatively, the sentinel animals were placed in a contaminated environment in absence of the infected donor animals (**Fig. 2A** and **B**). In the absence of antivirals, both strategies resulted in infection of the sentinel animals. Using this model, it was demonstrated that treatment of the donor or sentinel animals with the antiviral 2'-C-methylcytidine (2CMC) prevented transmission and reduced disease severity (**Fig. 2C**). Thus, the mice transmission model provides a valuable tool for future transmission studies and could be useful to address many of the current 'unknowns' of NV transmission, such as the relation between replication kinetics and transmission or the size and nature of genetic bottlenecks during NV transmission.

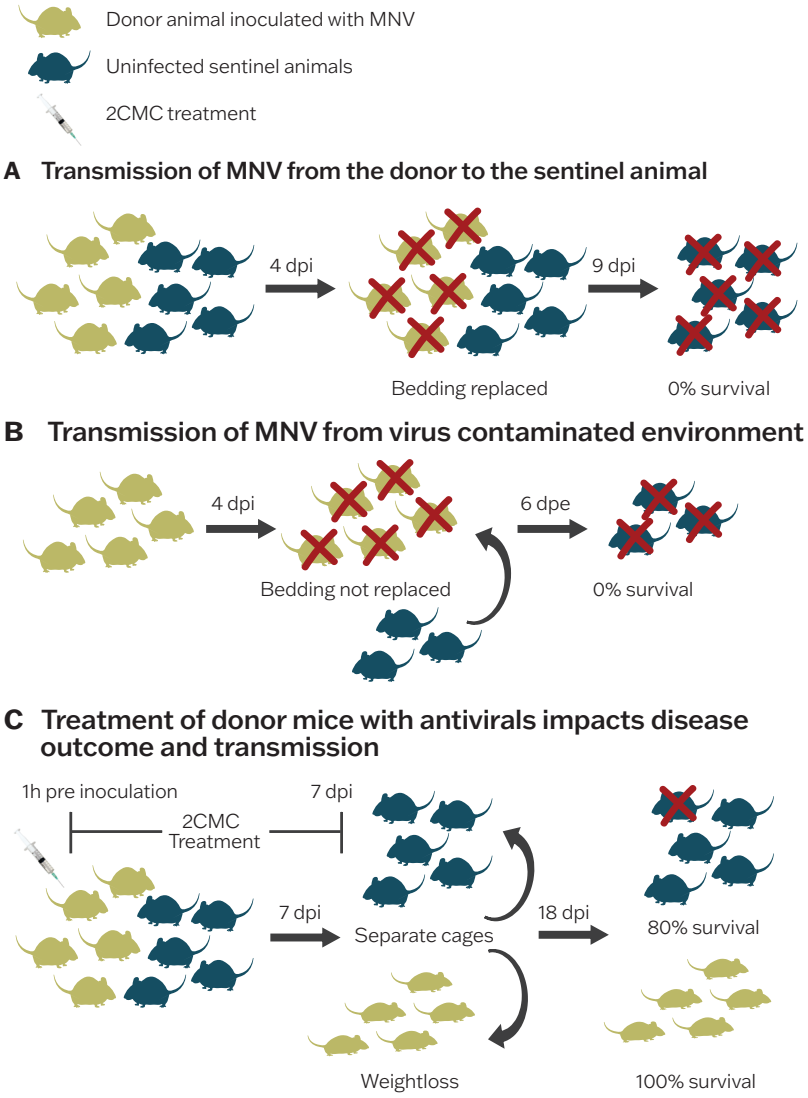

Figure 2. Mice model for contact transmission. Mice, deficient in INF-α/β and IFN-γ receptors were infected with MNV (**yellow**). Uninfected sentinel mice (**green**) were (**A**) placed with the infected donor mice or (**B**) in a contaminated environment in absence of the infected donor animals. Both settings resulted in infection of the sentinel mice. (**C**) Donor animals were treated with 2CMC (syringe) prior to inoculation with MNV and placed with sentinel animals. Seven days post inoculation (dpi), 2CMC treatment was discontinued and both groups were placed in separate cages. The 2CMC treatment had an impact on disease and transmission [538].

Clinical symptoms and transmission

It is likely that in the absence of clinical symptoms such as vomiting and diarrhea, transmission events are infrequent due to the lack of environmental contamination. Vomiting and toilet flushing can result in the formation of droplets and aerosols [539], and several studies have been dedicated to elucidate the role of vomiting in transmission through the airborne route and by environmental contamination. Initial indication for the possible role of vomiting in transmission came from outbreak investigations where the secondary attack rate of NV was inversely correlated to the distance of the contact to a person vomiting inside a confined space [540]. Human challenge studies with GI.1, GII.2 and GII.1 strains demonstrated that 40%-100% of the infected subjects vomited at least once. Most of the emesis samples contained detectable virus titers with mean titers of 8.0×10^5 and 3.9×10^4 genomic equivalent copies/ml for GI and GII viruses, respectively. A second factor is the severity of vomiting, described as projectile vomiting with abrupt onset. The force of emesis may influence on the dispersal of droplets and aerosols and thereby the severity of environmental contamination. To assess the extent to which an episode of projectile vomiting can contaminate the environment, a simulated vomiting system named Vomiting Larry was developed [541]. The model is based on the intragastric pressures, that reaches on average 10.93 kPa and can be as high as 38.66 kPa during vomiting, as measured during episodes of vomiting induced in volunteers by drinking Ipecac syrup [542]. Simulation studies with Vomiting Larry indicated that during an episode of projectile vomiting splashes and droplets can spread >3 m forward and 2.6 m lateral and that an area of at least 7.8 m^2 should be decontaminated [541]. Others were able to generate aerosols with the HuNV surrogate bacteriophage MS2 [543]. More importantly aerosolized HuNV genomes could be detected during outbreaks in health-care facilities with concentrations ranging from 1.4×10^1 to 2.4×10^3 genome copies per m^3 of air. That infectivity and integrity of NV particles can be preserved during aerosolization was shown with MNV [544]. Considering the low infectious dose needed to infect volunteers [66], these concentrations would likely be high enough to infect new hosts after inhalation and swallowing of the viral particles.

Stability in the environment

After shedding from the host NV particles have to remain stable in the environment prior to infecting a new host. The presence of bacteria can affect viral stability, for example binding of poliovirus to bacterial surface polysaccharides enhances virion stability [534, 545]. In the presence of bacteria MNV was more stable to electrical breakdown in water [546], while HuNV was found to be more stable to acute heat stress. Thus, the presence of bacteria might facilitate transmission owing to an increase in stability in the environment.

GII.4 strains have the highest prevalence in the winter season in temperate regions. For influenza it is thought that seasonality is related to stability and humidity as humidity can negatively affect transmission efficiency *in vivo* [547]. High humidity also resulted in a decrease of infectivity and binding capacity of murine and HuNV, respectively [548], while low humidity, like observed during the winter, was beneficial to NV survival. Although it should be noted that for non-GII.4 genotypes and in non-temperate regions seasonality is less clear [177]. Despite its sensitivity to humidity, HuNV is very stable in water. It was demonstrated that ground water spiked to a final concentration of ~6.5×10^7 GI.1 HuNV genomic equivalent copies/ml remained infectious to humans for at least 61 days. Remarkably, HuNV genome copies remained detectable in groundwater for over three years, although it was not assessed whether these represented infectious viruses [549]. In this study the infectivity was evaluated using human volunteers. The availability of the HuNV cell culture system will make it easier to determine what proportion of viruses that we can detect in the environment are infectious. Of interest, GI noroviruses have a higher association with waterborne infections compared to GII viruses and it is hypothesized that this is the result of a higher stability in water [29, 550], and limited removal efficiency during sewage treatment [551].

Conclusion

Despite major hurdles in culturing HuNV and the development of animal models, considerable progress has been made in understanding NV transmission. However, it is anticipated that the recent availability of cell culture systems and animal models will uncover many of the current 'unknowns' and will boost the development of vaccines, antivirals and treatment strategies. A better understanding of HuNV transmission and the development of outbreak control protocols and HuNV inactivation techniques will likely improve food safety and health-care.

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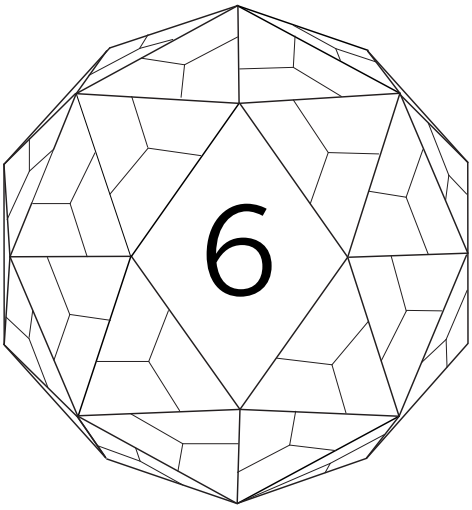


Summarizing discussion

Noroviruses are genetically diverse, with many genogroups and genotypes co-circulating in humans and animals. While the majority of outbreaks and sporadic infections are caused by GII.4 viruses, more than 40 genotypes have been described. A few genotypes are frequently detected, but the majority are only sporadically reported. Genotype GII.4 viruses are different from other genotypes as they undergo so-called epochal evolution, with a new variant emerging every few years. Despite the high sequence diversity between and within norovirus genotypes, within-host analysis of acute norovirus cases has shown minimal evolution during infection and was also limited during single outbreaks [22, 99, 111].

In addition to the new GII.4 variants, new ORF1, and ORF2 sequences frequently emerge in the human population. Newly emerged ORF1s that are found in combination with known capsids are termed "orphan sequences". One such example is the GII.P31 (previously GII.pe) polymerase. In which reservoirs these noroviruses evolved before they emerged in the human population is not known, but several potential reservoirs have been hypothesized. They include unsampled populations (e.g., asymptomatic individuals or people from demographic regions from which surveillance data are lacking), immunocompromised individuals in which noroviruses can evolve over several months, and animal reservoirs.

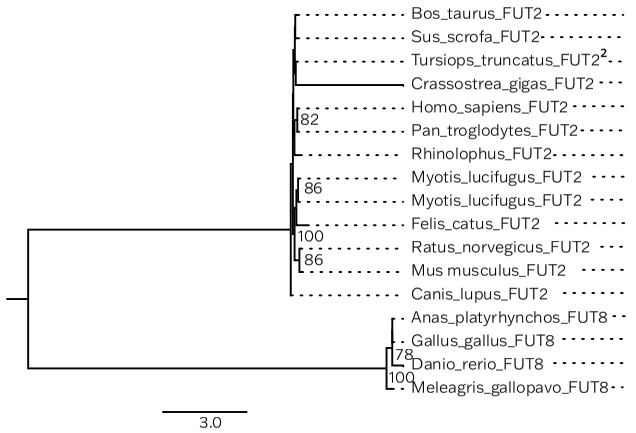
This thesis focuses on potential reservoirs of norovirus genotypes, variants, and recombinants and the mechanism that underlie the (re)-emergence of norovirus strains.

Noroviruses at the human-animal interface

Human norovirus transmission between animals and humans

Animals have long been proposed to be a potential norovirus reservoir due to the broad host range of noroviruses. For animals to play a role in human norovirus circulation, they need to be 1) in contact with either infectious hosts or a contaminated environment, 2) susceptible to the virus, and 3) subsequently transmit the virus to the next host.

In **Chapter 2.1**, we have performed a systematic review of data supporting or refuting norovirus transmission between animals and humans. Studies investigating the transmission of animal noroviruses to humans are scarce. In contrast, more studies described evidence supporting human-to-animal transmission of human noroviruses. Experimental infections demonstrated that several pigs and non-human primates can be infected with GI and GII genotypes leading to seroconversion and often resulting in diarrhea and virus shedding. None of these studies investigated subsequent animal-to-animal transmission, but in one experimental infection study, an infant macaque inoculated with GII.3 likely infected its co-housed mother [336].



Animal	Species-specific NoV	Susceptible (in lab) to human NoV	HBGA expression	VLP binding	Human NoV found in feces	Serological evidence[1]
Cow	+	+	+	NA	+	NA
Pig	+	+	+	+	+	+
Harbor porpoise	+	NA	+	+	NA	NA
Oyster	-	-	+	+	NA	NA
Human	+	+	+	+	+	+[3]
Non-human primates	-	+	+	+	+	+
Microbat	+	NA	+	+	-	NA
Cat	+	NA	+	+	-	NA
Rat	+	NA	+	+	+	NA
Mouse	+	+	NA	NA	-	NA
Dog	+	+	+	+	+	+
Mallard	-	NA	+	+	NA	NA
Chicken	-	NA	+	+	-	NA
Zebrafish	-	+	NA	NA	NA	NA
Turkey	-	NA	+	+	NA	NA
Gull	-	NA	-	-	+	NA
Megabat	-	NA	+	+	-	NA

Figure 1. Summary of norovirus susceptibility traits for different animal species. Maximum-likelihood tree of fucosyltransferase 2 and 8 (FUT2 and FUT8) nucleotide sequences from www.ensembl.org/index.html and Yamamoto et al. [284]. The tree was inferred by PhyML. += Positive; - =negative; NA=not available; 1 Antibodies against human noroviruses; 2 Bottlenose dolphin; 3 Antibodies against animal noroviruses.

And in one study, a dog infected with GII.4 by its owner, most likely subsequently infected its newborn pups [355]. A variety of human norovirus sequences have been found in animal feces, showing that animals shed human noroviruses outside controlled laboratory conditions, likely as a result of human-to-animal transmission. From the data that we summarized in **Chapter 2.1** most evidence suggests that dogs, pigs, and non-human primates may be a potential reservoir for human noroviruses (**Fig. 1**). These animals are not only regularly exposed to humans, but noroviruses have also been found in their feces, and antibodies against human noroviruses were detected in their sera. It should, however, be noted that many animals have not been included in serology studies and have not been tested regarding their susceptibility. Factors such as intensity of contact with humans will also impact the likelihood of transmission; For example, dogs are commonly found in households globally and are in close contact with humans, but primates are not common pets and their contact with humans is less intense.

In **Chapter 2.2**, we investigated potential transmission between humans and animals by phylogenetic analyses. To this end, we compared all human norovirus sequences detected in animal samples and compared them to strains circulating in humans. For most human norovirus sequences obtained from animal samples, the P2 domain was not covered and therefore amino acid changes in the HBGA binding site or antigenic epitopes could not be investigated. Only very few studies have sequenced the ORF1, the complete ORF2, or the complete genome. Of these viruses, some had identical amino acid sequences compared to strains circulating in humans, and it was therefore concluded that prolonged circulation of these strains in animals was unlikely. However, a GII.17 and a GII.7 that were detected in feces of captive rhesus macaques showed several amino acid changes in the capsid surface, located adjacent to or in antigenic epitopes, likely altering virus characteristics. The GII.17 was collected from a subsample of 50 fecal samples taken from animals at a monkey farm with ~2000 animals [260, 261]. And the GII.7 was detected in a colony of 500 macaques in a primate center of which 8.2% had tested PCR positive for GI, GII, or GIV [263]. The four GII.7 sequences from this outbreak were nearly identical (99%-100% nt similarity), indicating that these infections belonged to the same outbreak. The finding of GII.7 and GII.17 viruses that had accumulated several new amino acid substitutions implies a surveillance gap either in humans or in animals. While it has been established that human-to-animal transmission can occur, it remains to be seen whether viruses are transmitted back to humans. Also, direct human-to-animal transmission could only be demonstrated in a few studies as most of the studies did not include human samples [253, 355].

Zoonotic or reverse-zoonotic events can result in the adaptation of the virus to the new host [553, 554]. For noroviruses, it is not clear what adaptation would be necessary for successful emergence in a new host. Overall, there is limited information

for noroviruses about substitutions that result in phenotypical changes that could, among others, impact the norovirus host range. Most information has been acquired for the capsid which contains the receptor-binding site and antigenic epitopes. *In vitro* studies have shown that few amino acid substitutions are enough to alter Histo-blood group antigen (HBGA) binding [21] as well as changing antigenic properties [22, 103, 555]. Therefore, a few mutations could suffice to adapt the binding-specificity to a new host. Whether human norovirus infections in animals result in virus adaptation has only been investigated in a few non-human primate studies. In chimpanzees, experimentally inoculated with GI.1, at 12 to 34 days post-inoculation, amino acid substitutions had accumulated in the capsid close to the HBGA site but also in the RdRp [57]. In a binding experiment, the above-mentioned GII.17 (detected in feces from captive rhesus macaques) showed a significantly reduced binding signal to human saliva compared to the closest related human GII.17, probably a result of the amino acid substitutions on the capsid surface [260]. It was also reported that the rhesus GII.17 had a higher affinity for B positive saliva, which is the most common HBGA type in rhesus monkeys, indicating possible host adaptation.

In conclusion, we showed the close phylogenetic relatedness between human noroviruses that were found in animals and those found in humans. Human-to-animal transmission is a more likely scenario as the GII.4 variants were detected in animals after they had been circulating in the human population.

Norovirus attachment to animal and human tissues

While in humans the secretor status (dependent on the fucosyltransferase 2, FUT2) is an important determinant for norovirus binding and susceptibility, much less is known about its role in animals. Norovirus inoculation studies in pigs and non-human primates showed that while all infected animals were secretor positive, not all secretor animals were susceptible, indicating that additional factors may be important [57, 79, 268].

To further investigate the potential susceptibility of animal species to human noroviruses, we performed attachment studies. In **Chapter 2.3**, we tested norovirus attachment to animal and human intestinal tissues and showed that GI, GII, GIV, and GIX noroviruses attached to tissues of dogs, pigs, and chimpanzees. These were species, in which human noroviruses have been detected before and/or which are susceptible to experimental infection with human noroviruses. We also observed attachment to tissues of species in which no noroviruses have been found, such as the common pipistrelle and mallard. Although GII.4 is the genotype most commonly found in humans and animals, it was not the genotype with the broadest attachment pattern regarding species diversity. Instead, GI.3 and GI.6 showed the broadest attachment, binding to individuals of 10 and 13 of the 15 species tested, respectively. Attachment varied between individuals within the same species, suggesting that individual differences impact norovirus binding. We then

characterized the HBGA profile of these individuals to investigate if this could explain the variance in attachment, but differences in VLP attachment could not be explained by HBGA expression alone. However, while no clear correlation between expression of certain HBGAs and attachment of specific genotypes was detected, more norovirus genotypes attached to species that expressed a broader range of HBGAs. None of the genotypes attached to tissues of the black-headed gull, which was the only species in which none of the individuals expressed any of the tested HBGAs. We also found that GII genotypes did not attach to non-secretor individuals, indicating that secretor status is more important for GII than for other genogroups.

Our finding that VLPs also attached to tissues of some species for which no FUT2 encoding gene has been identified [284] raises the question of whether other attachment factors could play a role in these animals. The recent development of the zebrafish model that lacks a FUT2 implies that the importance of the secretor status for norovirus infection might be restricted to mammals, while in other animals different attachment factors could compensate [116]. For example, in zebrafish, FUT8 shares the highest similarity with FUT2 and it is also expressed in several other animals, for which no FUT2 homolog was detected (**Fig 1**).

In conclusion, the attachment of human noroviruses to tissues of a diverse array of animal species, implies that (human) noroviruses potentially have a broad host range. But note that the presence and attachment of viruses do not necessarily indicate replication and susceptibility.

Animal-to-human transmission

All recent pandemics have started with an animal-to-human transmission event [285, 553]. Therefore, the zoonotic potential of viruses and the linked risk of spillover events into the human population is critical for the study of emerging diseases. In contrast, the reverse, human-to-animal transmission, such as we observed for human noroviruses is less well studied. Some well-studied examples are the 2009 H1N1 and the COVID-19 pandemic, during which several events have been documented [556]. For the pH1N1 virus, 49 human-to-pig transmission events have been proposed but also to livestock turkeys and zoo animals [557, 558]. A recent example of reverse zoonosis followed by animal-to-human transmission is SARS-CoV-2, which was first introduced into a mink population and was then found to spill back into the human population [559]. Tracking of SARS-CoV-2 transmission between minks and humans was discovered due to a mink-specific amino acid substitution that could be found back in subsequently infected humans. This required carefully documented metadata and targeted Next-Generation Sequencing of numerous animal and human samples during the SARS-CoV-2 outbreak in minks. With the limited number of norovirus sequences obtained at the human-animal interface, such an event would almost certainly be missed.

Role of recombination in norovirus (re)-emergence

The study of transmission between humans and animals is also important because animal reservoirs could play a role in norovirus recombination. It has been speculated that some of the newly observed ORF1 or ORF2 sequences were obtained through recombination of human and animal strains. For example, genogroup GIX (former genotype GII.15) is genetically clearly distinct from GII viruses and closest related to the sea lion GNA2, while the ORF1 clusters together with GII viruses [3]. For influenza A, the emergence of animal viruses in humans has been linked to reassortment events, during which the genomes of human and avian influenza viruses, that consist of eight segments, combine to form a reassortant virus [560, 561]. Also, for rotaviruses, reassortant viruses containing segments of human, porcine or bovine origin have been described [562]. Although reassortment between gene segments is a different mechanism than recombination, it also requires co-infection of a single cell with two different virus strains. For influenza viruses, a well-known "mixing vessel" is pigs that get coinfected with human and avian influenza viruses [563]. For rotaviruses, it is not known where these reassorted viruses originate.

It is not well understood how new ORF1 and ORF2 sequences recombine with existing ORF1 and ORF2 sequences. It is also unclear why some ORF1/2 genotypes seem more restricted in their ability to recombine than others. For recombination to occur, several key steps are required. First, a host cell needs to be co-infected with more than one genotype or variant. Second, viral genomes of both viruses need to be replicated within the same cell. Third, the new recombinant virus then needs to be successfully transmitted to a new host and be competitive compared to parental viruses [445]. The exact mechanism underlying successful norovirus recombination is not known, but likely regulatory sequences of the ORF1 and ORF2 RNA sequences are important. Recombination has not been studied experimentally for human noroviruses and it has only been demonstrated *in vitro* for murine noroviruses by Mathijs *et al.* who provided evidence for homologous recombination between two distinct GV virus strains in cell culture [564].

Norovirus recombination is usually restricted to genotypes within one genogroup (intragenogroup) and between viruses that share high sequence similarity [445, 460]. The only reported intergenogroup recombinant viruses were a GI-GII [289], a feline and canine GVI-GIV recombinant [245], GVIII.1[GII.P28] [3], and the above-mentioned GIX. Therefore, recombination between animal and human noroviruses would most likely occur in a host that can be co-infected with human and animal norovirus genotypes that belong to the same genogroup. Porcine and feline/canine genotypes are the only known animal noroviruses that cluster in human norovirus genogroups, namely GII and GIV, respectively. In addition, pigs can be infected with human noroviruses, but to date, no recombinant norovirus genome consisting of human and known animal

norovirus genotypes has been detected. Another animal vessel that could contribute to the recombination of animal and human noroviruses is oysters. Co-infection of a host with several genotypes is regularly detected after shellfish-associated outbreaks [565-570], and animal noroviruses have been detected in oysters including a harbor porpoise norovirus that was most closely related to genogroup GI [62, 294, 373, 571].

In conclusion, based on experimental inoculations, surveillance data, and binding studies, a broad range of animals is potentially susceptible to human noroviruses. However, it is unclear how often these events occur and if they lead to subsequent circulation within the animal population. Our understanding of human-animal transmission events is hindered by the lack of full genome sequencing of human norovirus genomes detected in animals and matching human samples to determine the source of the virus and chains of transmission. Furthermore, genomic investigations of the evolution of human noroviruses in non-human hosts should be supplemented with phenotypical characterizations of key virus characteristics, such as binding specificity. As not all animals seem equally likely to play a role in norovirus transmission, species that are in close contact with humans should be prioritized and studies should ideally include animals and humans at the animal-human interface.

Norovirus emergence and circulation in humans

Of the more than 40 norovirus genotypes, the majority are only detected sporadically in outbreaks, indicating that current (outbreak-based) surveillance does not capture the full diversity of circulating viruses. An important indication that some genotypes might circulate undetected in the population is the high genotype diversity found in sewage surveillance. For example, GI viruses are, on average, found in <10% of reported infections (often in food-related outbreaks), but they are detected with disproportional high frequency in wastewater and sewage [356, 572, 573]. Some have hypothesized that these sporadically appearing noroviruses could be circulating undetected in children and that outbreaks are seeded from children into the adult population, where the majority of outbreaks are reported [574-576]. Alternatively, they could be circulating undetected in individuals with asymptomatic infections or immunocompromised patients. In **Chapter 3**, we investigated the role of children and immunocompromised individuals in norovirus circulation and evolution.

Development of a norovirus protein microarray

In **Chapter 3.1**, we investigated if we can detect serological traces of circulation of sporadically appearing genotypes in children. To this regard, we developed a protein microarray representing the P domains of the known human norovirus GI and GII genotype diversity. This protein array was further validated using pre- and post-infection sera of adults infected with a known norovirus genotype. We found

that while the highest IgG titers were directed against the genotypes of the infecting norovirus, post-infection titers also significantly increased against other genotypes from the same genogroup. In contrast to IgG, the IgA response was lower and more specific.

Profiling of humoral immune responses to norovirus in children

Children younger than five years and adults older than 65 years carry the largest burden of norovirus-associated gastroenteritis and associated deaths [37, 38]. By the age of two years, children probably have had ≥1 norovirus infection, and up to 36% of children were reported to have had >5 infections [577]. They have been reported to be infected by a high diversity of noroviruses including rare genotypes (e.g., GI.5, GI.7, and GII.23). Furthermore, GII.4 variants have been detected in children years before becoming the dominant variant globally [171].

To investigate if these sporadically appearing genotypes circulate more widely in children of a certain age than has previously been reported from standard surveillance, we tested 287 children's sera against >30 genotypes. Sera were collected from children up to the age of 5.5 years and we analyzed them in groups based on their presumed norovirus history status: newborns with maternal antibodies (0-6 months), naïve individuals (>6-12 months), individuals with presumed single or recent infection (>1-1.5 years) and individuals with presumed multiple infections (>1.5-5.5 years). For IgG we found an age-related pattern; In the first six months, 95.3% had antibodies against at least one genotype, followed by a drop after around six months and an increase in seroprevalence up to five years. In contrast, only 8.2% had IgA antibodies in the first six months and this steadily increased with age up to 41.2% in the oldest children. Very few children had antibodies against only one genotype and therefore we analyzed antibody responses that were significantly higher against a single genotype. These dominant IgG and IgA responses were directed against the genotypes that are most commonly found to cause outbreaks (GII.2, GII.3, GII.4, and GII.6). No sera that solely had a response towards rare genotypes were found, but we detected a few sera that recognized a limited number of antigens and had a strong response to a rarely detected genotype (i.e., GII.8, GII.14, and GII.23).

Few genotypes are associated with age and GII.3 is the best-known example, as it is more often found in outbreaks in children than in adults [97, 578]. Likewise, infections with GI viruses are not associated with certain age groups [34], but our data suggest that GI viruses could be more common in individuals older than five years. Our study showed comparable seroprevalence against GI and GII genotypes in the age group 0-6 months, which are likely maternal antibodies. These levels decreased in the age group >6-12 months and while the GII seroprevalence increased with increasing age, GI seroprevalence remained low until the age of five years. This could indicate that GII

genotype infections are on average more prevalent at a younger age and GI infections at a later age than included in this study. This has also been hypothesized by a study observing that seroprevalence against GI.1 increased in children once they attended school [413]. In conclusion, using the protein microarrays, we did not find serological evidence for the wide circulation of uncommon genotypes in children younger than 5.5 years.

Antibody responses against NS proteins

For noroviruses, there is frequent recombination between the ORF1, encoding the NS proteins, and ORF2, encoding the capsid protein. However, serological studies usually focus solely on antibodies directed against the capsid, as it is the most antigenic, but it provides little information on the seroprevalence of the polymerase genotypes.

Therefore, to investigate the exposure to ORF1, we integrated the NS proteins on the protein array in our study in **Chapter 3.1**. To assess which NS proteins are antigenic, we tested seroprevalence against all NS proteins in sera collected from 120 children. Antibodies were detected against all NS proteins except for the NTPase. The highest seroprevalence was detected against p48 (27.5%) and p22 (5.8%) and therefore we selected these two antigens to capture antibodies against a panel of ORF1 genotypes. We found a high seroprevalence in the 287 sera against the p48, but genotype-specific responses were rare and limited to a few genotypes (GI.P3, GII.P4, and GII.P7). Most of the sera with anti-NS antibodies were collected from individuals that likely had several norovirus infections, evident by high titers against several capsid genotypes. The seroprevalence was highest for GII.P31, GII.P4, and GII.P21, which are the ORF1 genotypes most commonly found in outbreaks. Whether these antibodies are functional remains to be investigated.

Antibodies against non-structural proteins have been described for other viruses, especially against secreted proteins in rotavirus and flavivirus infection. During rotavirus infection, the secreted form of the non-structural protein 4 (NSP4) acts as a virotoxin, which is important for the induction of diarrhea [579]. Antibodies against NSP4 were found in humans and animal models, and their protective effect against diarrhea has been shown in mice and macaques [580, 581]. In flaviviruses, NS1 is one of the main targets of antibodies and is therefore also a vaccine candidate. For murine noroviruses, p48 is cleaved into NS1 and NS2 [60], and immunization with NS1 provides protection against murine noroviruses by inhibiting infection of tuft cells, the main target cell type of some murine norovirus strains [60]. For human noroviruses, it should therefore be further investigated if the antibodies directed against p48 and p22 have an impact on disease outcome.

Development of antibody responses and cross-reactive antibodies

Serological studies have long been used to assess norovirus circulation. One benefit is that norovirus-specific antibodies can still be detected after the infection has cleared, however, with some known broadly reactive norovirus epitopes, cross-reactivity is a concern when investigating specific norovirus genotypes. Antibodies that can block the binding of norovirus to HBGAs show a more specific response [75, 85, 443], and similarly using only the most variable part of the capsid, the P domain, allows for the analysis of genotype-specific responses [417].

IgG cross-reactivity can be the result of either broadly reactive epitopes and/or reactivation of memory cells from previous infections. The idea of certain genotypes sharing cross-reactive epitopes is the basis for the premise of "immunotypes", which proposes that a primary infection protects from infection with genotypes from the same immunotype due to cross-protective epitopes [99]. In a recent study by Chhabra *et al.*, the risk of homo- and heterotypic reinfection was investigated [40]. The authors reported that children infected with GII.4 were less likely to get infected with GI.3 later on. While these two genotypes do not belong to the same proposed immunotype, this finding is an indication of potential cross-protective immunity between noroviruses from different genogroups.

Taken together, we reported here, for the first time the seroprevalence against the comprehensive diversity of human noroviruses. We did not find serological evidence for the wide circulation of less common genotypes. Surveillance studies often focus on children, and it is possible that while some genotypes are associated with infections in young children (e.g., GII.3), some genotypes might be more prevalent in adolescents and adults, causing sporadic acute gastroenteritis (AGE) or asymptomatic infections which are not often included in surveillance studies. This could be investigated by low-threshold testing after gastrointestinal symptoms in a cohort including individuals between five and 65 years or longitudinal testing of asymptomatic individuals. This would provide insights into the frequencies of sporadic and asymptomatic infections and associated genotypes. To better understand the development of the anti-norovirus antibody response, longitudinal studies, such as birth cohorts, should ideally include serological data. This would allow a better understanding of how antibody response develops regarding infecting genotypes and increase our understanding of norovirus cross-reactivity and possible cross-protection.

Norovirus emergence and circulation in immunocompromised patients

Immunocompetent individuals usually clear norovirus infections within days, although after waning of symptoms, prolonged shedding (>2 weeks) has been detected [130, 519, 582]. In contrast to healthy individuals, immunocompromised individuals can

become chronically infected for months to years [99, 107, 108, 110, 111, 452] and they have been of interest as they pose a reservoir for norovirus evolution and emergence in the general population.

Immunocompromised individuals as a source for norovirus diversity

In acute infections, the viral population is highly homogenous and this becomes more diverse when shedding is prolonged, either after symptoms have resolved or during chronic infections in immunocompromised individuals [99, 111]. During one year, 11 amino acid substitutions were detected in GII.3 in a chronically infected patient [106]. Of these substitutions, eight were detected in the P2 domain. Since then, several studies have investigated norovirus evolution in chronically ill patients and found fixation rates throughout the genome ranging from 0.01-0.21 amino acid changes/day [107, 108, 112]. These studies, including our data (**Chapter 3.2**), show that while mutations were detected throughout the whole genome, the dN/dS ratio was highest in the P2 domain, indicating that there is positive selection rather than drift alone, which suggests some level of immune pressure. Although our study included mostly transplant recipients that take immunosuppressive drugs, immunocompromised patients encompass a broad spectrum of individuals with varying degrees of immune impairment, like individuals with primary immunodeficiencies, cancer patients, and HIV-positive people. Many of these individuals will have some level of antibody response, but the existence of post-infection antibodies has only been demonstrated in two studies [168, 169]. An in-depth understanding of the immune response to norovirus infections in immunocompromised patients and how it develops throughout a chronic infection is lacking, and the link between immune response and intrahost evolution is therefore unknown. Interestingly, Siebenga *et al*. found that the rate of mutation fixation was negatively associated with the severity of immune impairment; in the patient with no humoral immunity, the fixation rate was lower compared to patients with a properly functioning immune system [108]. This is of interest concerning the risk these viruses pose to the population.

Immunoglobulin treatment in chronically infected immunocompromised patients

Chronically infected patients can suffer from prolonged diarrhea and associated weight loss, but the treatment options in these patients are limited and are often restricted to off-label prescription of antivirals or oral admission of immunoglobulins (Ig) [160-163, 166, 167]. These Ig preparations are prepared from plasma collected from numerous donors to ensure diverse specificities of antibodies against a broad spectrum of pathogens. These interventions vary in their success rate. The reason why the treatment works for some patients and not for others is not yet understood. To better understand how intra-host evolution in immunocompromised patients could influence

Ig treatment, we investigated the effect of the accumulated amino acid substitutions on the blocking ability of Igs (**Chapter 3.2**). Overall, we found that the noroviruses found in chronically infected immunocompromised patients, over time accumulated amino acid changes in the VP1, which in some patients reduced or eliminated the ability of Igs to block VP1 binding. This shows that prolonged intr-ahost evolution likely reduced the effect of Ig treatment. In theory, this could mean that the success rate of this treatment is highest when given as early as possible before the virus diverges too much from circulating strains.

Risk for the general population

Transmission from immunocompromised shedders to other immunocompromised or immunocompetent individuals has been documented [106, 110, 583]. However, to date, none of the viruses identified in immunocompromised individuals has become a widely circulating variant and when compared to norovirus diversity in the population, these viruses are genetically distinct as shown in this thesis and other studies [107, 466]. We showed that after prolonged replication in an immunocompromised host, norovirus binding was no longer blocked by antibodies present in Ig preparations, which represent the antibody spectrum of the population. This indicates that viruses evolving in immunocompromised patients could in theory evade immunity in the general population, similar to new GII.4 variants that are detected every few years. Also, in another study within-host evolution of a GII.4 strain resulted in a significant decrease in blocking titers of polyclonal mouse sera [114].

To investigate the risk of these viruses causing large outbreaks in an immunocompetent population, other factors than immune evasion remain to be investigated. But research on these viruses was restricted for decades by the lack of culture models. Recently, replication of noroviruses from immunocompromised patients in cell culture and a zebrafish model were reported [116, 584]. In these studies, viruses from earlier time points replicated often, but not always, more efficiently than viruses taken after several weeks or months of intrahost evolution, indicating reduced fitness in cell culture [584]. In concordance, we showed here that after prolonged infection the ability of the virus to attach to HBGA-containing saliva or pig mucin was reduced. The impact of this decrease in binding requires further investigation. With the recent availability of these systems, we can now start to characterize these viruses *in vitro* and *in vivo*.

As Ig treatment has been administered successfully in several cases, it remains to be seen how this could impact virus evolution. During the COVID-19 pandemic, some SARS-CoV-2 variants have arisen that differed by several amino acid substitutions, in contrast to the limited number of changes that have been detected before. These variants have been proposed to have evolved in immunocompromised patients treated with convalescent plasma [585]. The COVID-19 pandemic has also demonstrated that

even though sequencing data for SARS-CoV-2 was magnitudes higher than for any other virus, the origin of new variants is still not well understood. This raises the question of whether a drastic increase in surveillance and higher coverage of sequencing would be enough to track new GII.4 variants before their global emergence.

In conclusion, the main argument against the role of these viruses in global norovirus circulation is that none of the GII.4 variants emerging in the human population could be traced back to an immunocompromised patient. However, considering that the study of chronic shedders is often limited to a few individuals, it is likely that the origin of a new GII.4 variant from such an individual would be missed.

The role of the polymerase for norovirus emergence

The VP1 is by far the best-studied norovirus protein, and especially for GII.4, the antigenic evolution of the capsid and HBGA binding specificity have been well studied [103, 450, 461, 586]. Some recombinant viruses with very similar capsids but different ORF1s show high divergence in their prevalence implying that the ORF1 could add to the prevalence of certain recombinants. The emergence of the recombinants GII.4[P4] in the late 1980s, GII.4[P31] in 2012, GII.4[P16] in 2016, and GII.17[P17] in 2015 are of special interest as their (re)-emergence was associated with the acquisition of a new polymerase (RdRp) [587].

Differences in RdRp activity

Up to the year 2012, every few years the dominant GII.4 variant was replaced by a new, antigenically different capsid variant. Since 2012, however, the GII.4 Sydney 2012 capsid has not been replaced. Instead, in 2016, the GII.2[P16] emerged which up to that date had been rarely detected. The GII.2 capsid did not show any amino acid substitutions implying that antigenicity should be similar to GII.2 viruses circulating at low levels for decades [497]. The GII.P16 polymerase, however, showed four amino acid substitutions compared to the GII.P16s that were detected before 2016 [497]. The same GII.P16 was also found associated with the GII.4 Sydney 2012 capsid [498]. The circulation of these two viruses, which showed no antigenic drift in their capsid but a polymerase with distinct amino acid changes led to the hypothesis that their emergence could be due to new characteristics of the RdRp, rather than the capsid. Increased RdRp activity could result, for example, in a higher viral load, which could subsequently lead to more shedding and a higher transmission rate.

In **Chapter 4**, we investigated the RdRp activity of a panel of GII.P16 and non-GII.P16, some of which are commonly found and some that are rarely detected. To compare polymerase activity we used two assays, a cell-based luciferase reporter assay, and a cell-free system. While we found differences in polymerase activity between the selected RdRp, we could not detect differences that explained the recent prevalence

of the new GII.P16 RdRp lineage over the old GII.P16 RdRps lineages. Furthermore, levels of GII.P16 polymerase activity were comparable to other frequently detected RdRps such as GII.P31 and GII.P4 as well as some RdRps that are rarely found like GII.P2.

The VP1 can have a stimulatory effect on RdRP activity (personal communication Ian Goodfellow), and therefore we tested several RdRp-capsid combinations. Some capsids had a stronger effect on polymerase activity than others but this effect was also observed for RdRp-capsid combinations that have never been detected. Although it does not explain why some RdRp-capsid combinations are more frequently found than others, it does suggest that these two proteins interact.

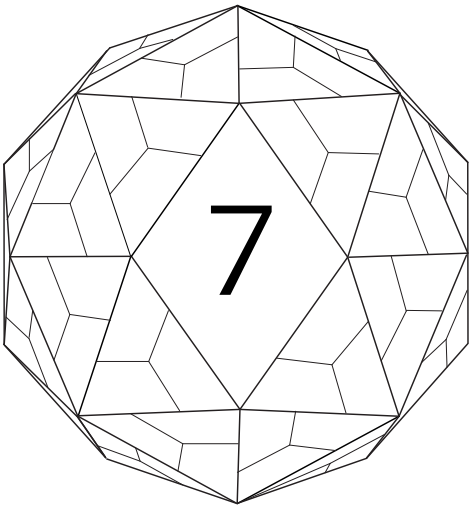
RdRps have been compared before regarding their incorporation rate and/or mutation rate, which has been investigated using biochemical assays [502]. GII.P7, which is infrequently detected, had a higher incorporation rate than the prevalent GII.P4 but GII.P4 had a higher mutation rate that translated into an average of six nucleotide substitutions/capsid/year, while this was only 1.5 substitutions for GII.3 and 0.17 for GII.7 [502]. This shows that a higher activity does not necessarily result in a higher mutation rate, as has been suggested for other viruses [588]. These assays provide data on polymerase activity but not in the context of the whole virus. When the evolutionary rates of the capsids were compared between genotypes, most genotypes were static, with very few amino acid changes accumulating in the capsid. GII.4 and one GII.17 lineage were the only genotypes with evolving patterns [99]. Interestingly, when different GII.17 lineages were analyzed separately, the old lineages showed low sequence diversity during 37 years, while the emerging lineages from 2014/15 had diverged 5.4% in amino acid sequence within one year [99]. This indicates that genotypes that are considered static genotypes now, could potentially become evolving genotypes when associated with a new RdRp. One factor that should, however, be acknowledged is the possibility that different genotypes have varying tolerance for alteration within the capsid structure, as has been proposed by Donaldson *et al.* [4]. If true, norovirus evolution can be seen as a result of the RdRp mutation rate and the tolerance of the capsid toward amino acid changes and possible subsequent alterations in the capsid structure.

Analyzing RdRp mutation rates *in vitro* provides valuable information about the inherent polymerase qualities, but they are not isolated from other characteristics of the virus. Norovirus polymerase has rarely been investigated *in vivo*, except for the murine norovirus (MNV) RdRp. Arias *et al.* have demonstrated that a high-fidelity MNV RdRp mutant led to a three-fold lower virus diversity and showed delayed replication *in vivo* as well as a decrease in transmission in a mouse model [589]. In cell culture, these effects were not detected. In line with other RdRp studies, this implies that virus diversity is important *in vivo* and that the effect of a reduced virus diversity might not be detectable *in vitro* propagation [590].

Concluding remarks

The known diversity of norovirus is continuously expanding, and many questions regarding this diversity remain. It is still under debate in which reservoirs these viruses evolve, and we have investigated several possibilities in this thesis. To better understand this diversity and norovirus circulation, additional surveillance, targeting individuals that are currently missed by standard surveillance, is needed. These studies should ideally include animals and humans at the animal-human interface as not all animals seem equally likely to play a role in norovirus transmission. A better understanding of norovirus circulation is also needed for individuals that do not seek medical attention due to mild or lack of symptoms, as well as populations that live in areas where surveillance is sparse.

At the same time, there is a need for a better understanding of phenotypical changes that are linked with norovirus (re)-emergence. To comprehend norovirus (re)-emergence, research should not only include identification of the host and potential reservoirs but also how noroviruses spread from host to host. In **Chapter 5**, we have reviewed the currently available tools to study norovirus infection and transmission, including the more recently developed cell culture systems and animal models, that have opened the door to address new research questions. For example, comparing inherent virus characteristics, such as replication efficiency could help address the question of why some norovirus genotypes are more frequently detected than others. Furthermore, efforts should be put into the development of a reverse genetic system for human noroviruses. This will allow detailed investigations on the role of the polymerase and other non-structural proteins and the impact of amino acid substitutions and recombination in the backbone of a complete virus. Together, a better understanding of the current diversity of noroviruses and the mechanism that underlies emergence on novel strains will aid to improve prevention.



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Authors' affiliations

Annelies Kroneman
National Institute for Public Health and the Environment (RIVM), Bilthoven, The Netherlands

Brenda M. Westerhuis
Department of Viroscience, Erasmus Medical Centre, Rotterdam, The Netherlands

Carlo Giaquinto
Division of Paediatric Infectious Diaseases, Department of Women's and Children's Health, University Hospital of Padua, Padova, Italy

Claudia M.E. Schapendonk
Department of Viroscience, Erasmus Medical Centre, Rotterdam, The Netherlands

Dennis A. Hesselink
Erasmus MC Transplant Institute, Rotterdam, The Netherlands

Department of Internal Medicine, Division of Nephrology and Transplantation, Erasmus MC, Rotterdam, The Netherlands

Erwin de Bruin
Department of Viroscience, Erasmus Medical Centre, Rotterdam, The Netherlands

Felicity Chandler
Department of Viroscience, Erasmus Medical Centre, Rotterdam, The Netherlands

Georgina I. Aron
Department of Viroscience, Erasmus Medical Centre, Rotterdam, The Netherlands

Harry Vennema
National Institute for Public Health and the Environment (RIVM), Bilthoven, The Netherlands

Herman Goossens
Laboratory of Medical Microbiology, Vaccine & Infectious Disease Institute (VAXINFECTIO), University of Antwerp, Antwerp, Belgium

Ian G. Goodfellow
Division of Virology, Department of Pathology, University of Cambridge, Cambridge, UK

Janko van Beek
Department of Viroscience, Erasmus Medical Centre, Rotterdam, The Netherlands

Jenny I.J. Meier
Department of Viroscience, Erasmus Medical Centre, Rotterdam, The Netherlands

Jeroen J.A. van Kampen
Department of Viroscience, Erasmus Medical Centre, Rotterdam, The Netherlands

Judit Szarvas
Research Group for Genomic Epidemiology, Division for Global Surveillance, National Food Institute, Technical University of Denmark, Kongens Lyngby, Denmark

Julia A. Bielicki
Paediatric Infectious Diseases Research Group, Institute for Infection and Immunity, St George's University of London, London, UK

Department of Infectious Diseases and Vaccinology, University of Basel Children's Hospital (UKBB), Basel, Switzerland

Kevin Groen

Department of Viroscience, Erasmus Medical Centre, Rotterdam, The Netherlands

Louise Sigfrid
Centre for Tropical Medicine and Global Health, Nuffield Department of Medicine, University of Oxford, Oxford, UK

Malte Kohns Vasconcelos
Paediatric Infectious Diseases Research Group, Institute for Infection and Immunity, St George's University of London, London, UK

Institute for Medical Microbiology and Hospital Hygiene, Heinrich Heine University Düsseldorf, Düsseldorf, Germany

Marion P.G. Koopmans
Department of Viroscience, Erasmus Medical Centre, Rotterdam, The Netherlands

Miranda de Graaf
Department of Viroscience, Erasmus Medical Centre, Rotterdam, The Netherlands

Myra Hosmillo
Division of Virology, Department of Pathology, University of Cambridge, Cambridge, UK

Pieter L.A. Fraaij
Sophia Children's Hospital, Erasmus Medical Centre, Rotterdam, The Netherlands

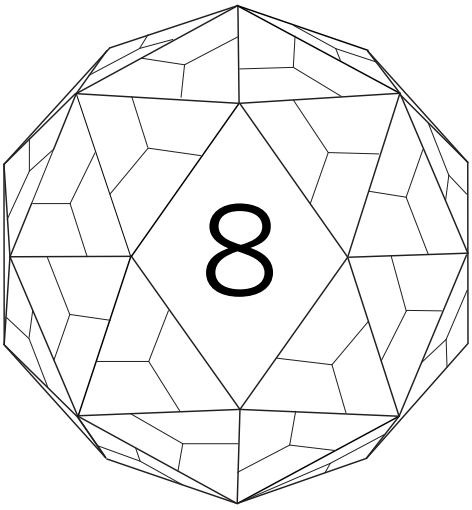
Ray W. Izquierdo-Lara
Department of Viroscience, Erasmus Medical Centre, Rotterdam, The Netherlands

Tran T.N. Thao
Institute of Virology and Immunology (IVI), Bern, Switzerland

Department of Infectious Diseases and Pathobiology, Vetsuisse Faculty, University of Bern, Bern, Switzerland

Department of Microbiology, Blavatnik Institute, Harvard Medical School, Boston, Massachusetts, USA

Virgil A.S.H. Dalm
Department of Internal Medicine, Division of Clinical Immunology, Erasmus University Medical Center, Rotterdam, The Netherlands



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Samenvatting

Norovirussen zijn in de jaren tachtig geïdentificeerd als de veroorzakers van uitbraken van acute gastro-enteritis (AGE). Ze behoren tot de familie van de *Caliciviridae*. Deze omvat virussen die zoogdieren, vogels en vissen infecteren. Het virus bestaat uit een buitenste schil, het "capside" genoemd, deze is samengesteld uit 180 kopieën van het VP1 eiwit. Op basis van genetische verschillen in het VP1 worden norovirussen in 10 genogroepen (GI-GX) ingedeeld die verder in 48 genotypen worden onderverdeeld. Mensen kunnen geïnfecteerd worden door norovirussen die tot genogroep GI, GI, GIV, GVIII en GIX behoren. De andere norovirusgenogroepen worden aangetroffen bij landbouwhuisdieren (bijvoorbeeld koeien en varkens), huisdieren (bijvoorbeeld honden en katten) en wilde dieren (bijvoorbeeld muizen, bruinvissen en vleermuizen). Ondanks deze grote verscheidenheid aan norovirussen zijn de virussen van genotype GII.4 de meest voorkomende norovirussen bij de mens. Net als bij influenzavirussen evolueren GII.4-norovirussen stapsgewijs, waarbij oude varianten worden vervangen door nieuwe. In welke gastheren deze virussen evolueren tot een nieuwe variant is niet bekend. Sommige niet-GII.4 norovirussen worden zelden gerapporteerd en het is onduidelijk of deze virussen alleen in mensen circuleren of dat ze ook in andere gastheer reservoirs circuleren tijdens de periodes dat ze niet in de bevolking worden gedetecteerd. In dit proefschrift hebben we de rol van dieren en mensen in de evolutie en circulatie van norovirussen onderzocht.

Norovirus verspreiding op het raakvlak tussen mens en dier

In **Hoofdstuk 2** hebben we de mogelijkheid onderzocht dat dieren een rol spelen bij de circulatie van norovirussen. Verschillende studies hebben dit onderzocht en we hebben deze bevindingen samengevat in **Hoofdstuk 2.1**. We ontdekten weinig bewijs dat dierlijke norovirussen mensen op natuurlijke wijze infecteren. Daarentegen kunnen ten minste zeven diersoorten experimenteel met humane norovirussen worden geïnfecteerd en in verschillende studies werd het genetisch materiaal van humane norovirussen in dierlijke ontlasting aangetoond. In **Hoofdstuk 2.2** hebben wij de in dieren gevonden humane norovirussen vergeleken met de norovirussen die in mensen circuleren. Deze vergelijking stelt ons in staat om in te schatten hoe lang deze virussen al in dieren circuleren. We weten dat genetische wijzigingen, die leiden tot veranderingen in het VP1 eiwit (vooral het P domain), de viruskenmerken kunnen beïnvloeden, aangezien de structuur van dit eiwit bepaalt hoe het virus zich aan het celoppervlak van de gastheer hecht, en omdat het herkend wordt door het immuunsysteem van de gastheer. Veranderingen in dit eiwit kunnen enerzijds de manier veranderen waarop het virus de gastheercel herkent en anderzijds leiden tot immuunontwijking, wat betekent dat norovirussen niet langer door het immuunsysteem van de gastheer herkend en geëlimineerd kunnen worden. Toen wij de genetische kenmerken van humane norovirussen bij dieren onderzochten, vonden wij verschillen tussen GII.7 en

GII.17 die in makaken waren gevonden en GII.7 en GII.17 die in mensen circuleerden. Het is mogelijk dat deze virussen zich aan hun dierlijke gastheer hebben aangepast. De accumulatie van mutaties (veranderingen in het genetisch materiaal) impliceert dat deze virussen onopgemerkt in dieren of mensen circuleerden.

Elke infectie begint met binding van het virus aan de gastheercel. Vervolgens dringt het virus de cel binnen en zet de replicatiecyclus in gang, die vervolgens resulteert in het vrijkomen van virusdeeltjes die nieuwe cellen kunnen infecteren. Welke moleculen op gastheercellen worden herkend, hangt af van het specifieke virus. Norovirussen hechten zich aan een groep suikers, gezamenlijk histo-bloedgroepantigenen (HBGA's) genoemd. Deze worden aangetroffen op het oppervlak van darmcellen en worden ook uitgescheiden in speeksel. Voor mensen is aangetoond dat de aanhechting van norovirussen aan speeksel of weefsels van het maagdarmkanaal (waar het norovirus infecteert) correleert met de vatbaarheid van een individu voor norovirussen. Om te testen welke diersoorten mogelijk geïnfecteerd kunnen worden door humane norovirussen, onderzochten we de bindings specificiteit van norovirussen aan darmweefsels van verschillende diersoorten (**Hoofdstuk 2.3**). We vonden dat verschillende humane norovirus-genotypes hechtten aan darmweefsels van varkens, honden, dwergvleermuizen, chimpansees en wilde eenden. Daarentegen hechtten minder genotypen aan darmweefsels van katten, ratten, bruinvissen, kalkoenen, kippen en oesters. Dee enige twee darmweefsel waaraan geen van de humane norovirus genotypen aanhechtte waren die van kokmeeuw en palmvleerhond.

Er zijn vele soorten HBGA's en de verdeling ervan verschilt van mens tot mens. Op basis van deze verschillen zijn sommige individuen resistent tegen infectie met bepaalde norovirus-genotypes. Aangezien deze correlatie nog niet bij dieren is bestudeerd, onderzochten wij of het hechten van norovirussen aan dierlijk weefsels geassocieerd was met hun HBGA-types en distributie. HBGA's werden gedetecteerd in de darmweefsels van de meeste diersoorten en individuen, maar we vonden geen direct verband tussen een specifiek HBGA-type en de binding van een specifiek norovirus genotype. Virussen van de GII genogroep bonden aan minder weefsels dan virussen van de GI genogroup, wat impliceert dat GII-virussen specifieker zijn in welke HBGA's ze kunnen binden dan GI-virussen. Op basis van het brede scala van diersoorten dat HBGA in de weefsels van het maagdarmkanaal heeft, zou het dierlijke gastheerbereik van humane norovirussen breed kunnen zijn. Het is echter waarschijnlijk dat nog andere factoren de vatbaarheid voor norovirus infectie beïnvloeden.

De rol van de mens in de evolutie en verspreiding van norovirussen

In **Hoofdstuk 3** hebben we gekeken naar de rol van de mens in de virusverspreiding en evolutie van norovirussen. De ziektelast en het sterftecijfer ten gevolge van norovirussen zijn het hoogste bij kinderen jonger dan vijf jaar en mensen ouder dan 65

jaar. Aangezien bij kinderen een grote diversiteit aan norovirussen werd aangetroffen, werd verondersteld dat bepaalde norovirus-genotypes die slechts sporadisch worden gerapporteerd, onopgemerkt in kinderen circuleren. In **Hoofdstuk 3.1** hebben we deze hypothese onderzocht door het antilichaamrepertoire van kinderen van 0-5 jaar te analyseren. Elke infectie, die het immuunsysteem activeert, leidt tot de productie van antilichamen tegen de infecterende ziekteverwekker. Deze pathogeen-specifieke antilichamen kunnen nog maanden tot jaren na de infectie in het bloed van een individu worden opgespoord. Het karakteriseren van de in het bloed aanwezige antilichamen wordt daarom gebruikt om infecties die een persoon heeft doorgemaakt, op te sporen. Met behulp van een eiwit-microarray testten we antilichamen in sera van ~300 kinderen tegen het VP1 P domein van ~30 humane norovirus-genotypes. Wij onderzochten zowel immunoglobuline G (IgG), het meest voorkomende type antilichaam in het bloed, als ook immunoglobuline A (IgA), dat in lagere concentraties aanwezig is in bloed maar het meest overvloedig aanwezig is in speeksel en mucus. Meer dan 90% van de sera van de jongste kinderen (0-6 maanden) hadden IgG dat ten minste één norovirus genotype herkende. Dit zijn waarschijnlijk antilichamen van de moeder die tijdens de zwangerschap de placenta passeren om pasgeborenen gedurende de eerste zes maanden tegen infectie te beschermen. In sera van kinderen ouder dan 6 maanden daalde het IgG-niveau van de norovirus-specifieke IgG antilichamen, wat erop wijst dat de maternale antilichamen waren afgenomen. De maternale antilichamen zijn hoofdzakelijk IgG en bij kinderen jonger dan 6 maanden vonden we slechts enkele sera met norovirus-specifieke IgA, maar deze aantallen namen toe met de leeftijd. In sera van oudere kinderen (>1 jaar) namen de IgA-spiegels toe en waren het hoogst bij de oudste kinderen (5,5 jaar). Dit wijst erop dat kinderen in de eerste levensjaren een eerste norovirus infectie doormaken.

Veel sera bevatten IgG dat meer dan één norovirus genotype herkende, waarschijnlijk als gevolg van kruisreactiviteit. Dit betekent dat antilichamen verschillende genotypes herkennen die overeenkomsten vertonen in regio's die door antilichamen worden herkend. Het was daarom onmogelijk te bepalen met welke genotypen deze kinderen eerder geïnfecteerd waren gewest. Om dit te omzeilen, analyseerden wij vervolgens alleen sera die een significant hoger signaal vertoonden tegen één genotype. Deze antilichamen herkenden genotypes die het vaakst norovirus uitbraken veroorzaken. Dit betekend dat de standaard surveillance representatief is voor de circulatie van humane norovirussen bij kinderen.

Een andere groep die mogelijk van belang is voor de evolutie en verspreiding van norovirussen zijn mensen met een immuundeficiëntie. Bij gezonde personen duren acute norovirus infecties slechts enkele dagen en het virus wordt vaak niet langer dan twee weken uitgescheiden. Bij mensen met een verzwakt immuunsysteem kunnen norovirus infecties chronisch worden en dus jaren voortduren, waarbij patiënten continue norovirussen uitscheiden in hun ontlasting. Tijdens deze langdurige infecties

accumuleren norovirussen mutaties waardoor ze genetisch verschillen van norovirussen die in de gezonde populatie circuleren. Er bestaat nog geen goedgekeurde behandeling voor deze patiënten, maar een experimentele behandeling die in verscheidene gevallen succesvol is gebleken, is de orale toediening van immunoglobuline preparaten (Ig). Deze preparaten bevatten antilichamen uit het bloed van >1000 volwassenen en bevatten dus het gemiddelde antilichaam spectrum van de volwassen bevolking. Ig-preparaten herkennen en binden zich aan het VP1-eiwit waardoor de binding van het virus aan de HBGA's op de darmcellen, wordt geblokkeerd en daardoor de infectie wordt geremd. Bij sommige patiënten leidde deze behandeling tot een succesvolle eliminering van de norovirus infectie, terwijl voor andere patiënten deze therapie de infectie niet oploste. In **Hoofdstuk 3.2** onderzochten we een mogelijke reden voor deze verschillen in het succes van de Ig behandeling. Daarvoor bepaalden we de genetische kenmerken van de norovirussen in chronisch geïnfecteerde patiënten. Gedurende enkele maanden accumuleerden zich mutaties, met name in het P domein van het VP1-eiwit dat belangrijk is voor de binding van het norovirus en de herkenning door het immuunsysteem. Bij sommige patiënten kon de binding van deze virussen niet langer worden geblokkeerd door de antilichamen in de Ig-behandeling, waardoor het effect van de behandeling waarschijnlijk afneemt. Dit zou kunnen verklaren waarom sommige patiënten na behandeling niet genezen en suggereert dat de kans op succes groter is als Ig's in een vroeg stadium van de infectie worden gegeven.

De rol van de polymerase in de evolutie en verspreiding van norovirussen

In **hoofdstuk 4** hebben we een mogelijk mechanisme onderzocht dat van invloed zou kunnen zijn op de verspreiding en prevalentie van nieuwe norovirussen. Wanneer een individu gelijktijdig met twee verschillende norovirussen geïnfecteerd raakt, kan er genetisch materiaal tussen deze virussen uitgewisseld worden (recombinatie). Voor norovirussen is aangetoond dat recombinatie plaatsvindt tussen het open leesraam (open reading frame, ORF) 2 dat voor het VP1-eiwit codeert en het ORF1 dat voor de niet-structurele eiwitten codeert. Deze niet-structurele eiwitten maken geen deel uit van het virusdeeltje maar spelen een belangrijke rol in de cel tijdens de infectiecyclus. Humane norovirussen hebben zes niet-structurele eiwitten en één daarvan is het RNA-afhankelijke RNA-polymerase (RdRp). Het RdRp is het enzym dat belangrijk is voor de replicatie van het virale genoom in de cel. Een hogere RdRp-activiteit zou kunnen leiden dat in een gastheer een groter aantal virussen wordt geproduceerd en dat bijgevolg meer virussen worden uitgescheiden, waardoor de kans op verspreiding naar een andere gastheer toeneemt. Wij hebben de RdRp-activiteit van verschillende norovirussen vergeleken, en hoewel de RdRp's in activiteit verschilden, was dit niet voorspellend voor de frequentie waarmee een bepaald norovirus bij uitbraken werd gemeld. Sommige RdRp-VP1 combinaties worden bijzonder vaak gemeld bij uitbraken, en bij het meten van de RdRp-activiteit in aanwezigheid van verschillende

VP1-genotypen vonden we dat sommige combinaties resulteerden in een hogere RdRp-activiteit dan andere. Dit effect was niet genotype-specifiek en RdRp-VP1 combinaties die vaak worden aangetroffen, resulteerden niet noodzakelijk tot een hogere RdRp-activiteit dan combinaties die minder vaak worden gemeld. Hieruit concludeerden we dat het RdRp mogelijk een rol zou kunnen spelen bij de verspreiding en prevalentie van norovirussen, maar de precieze rol nog verder onderzocht moet worden.

Conclusie

Norovirus onderzoek is afhankelijk van moleculaire systemen en technieken en in **Hoofdstuk 5** hebben wij de thans beschikbare technieken beschreven die nodig zijn om inzicht in de overdracht van norovirussen van gastheer op gastheer te verbeteren. Celkweek modellen zijn van cruciaal belang voor het karakteriseren en vergelijken van verschillende norovirussen met betrekking tot replicatie kinetiek en virus-gastheer interacties. Bovendien zijn diermodellen waardevol voor de studie van cel tropisme, pathologie en virusoverdracht.

Het in dit proefschrift beschreven onderzoek draagt bij tot de huidige kennis over potentiële reservoirs van circulerende norovirussen. De gepresenteerde resultaten suggereren dat dieren en individuen met een immuundeficiëntie hier ook een rol in kunnen spelen, en beide moeten in dat verband verder worden onderzocht. Voorts moet de norovirus surveillance ook populaties includeren, die momenteel door standaard surveillance worden gemist, zoals verschillende diersoorten en asymptomatische met norovirus geïnfecteerde personen. Hiervoor zou ook de in dit proefschrift beschreven eiwit-microarray ingezet kunnen worden om de blootstelling aan humaan norovirus te meten. We beschrijven ook dat het polymerase zou kunnen bijdragen tot de snelle verspreiding van sommige norovirussen maar niet tot de verspreiding van andere. Hoewel het VP1-eiwit tot nu het meest uitgebreid is bestudeerd van de norovirus-eiwitten, stellen wij voor dat de rol van de niet-structurele eiwitten bij het ontstaan van het virus verder moet worden onderzocht. Uiteindelijk is inzicht in het reservoir en de moleculaire mechanismen die ten grondslag liggen aan het ontstaan van norovirussen van cruciaal belang om op de juiste wijze te kunnen ingrijpen in de verspreiding van norovirussen.

Zusammenfassung

Noroviren wurden in den 1980er Jahren als Erreger von Ausbrüchen der akuten Gastroenteritis (AGE) identifiziert. Sie gehören zur Familie der Caliciviridae, zu der Viren gehören, die Säugetiere, Vögel und Fische infizieren. Das Virus besteht aus einer äußeren Hülle, dem sogenannten Kapsid, das aus 180 Kopien des VP1-Proteins zusammengesetzt ist. Basierend auf den genetischen Unterschieden in VP1 werden Noroviren in 10 Genogruppen (GI-GX) eingeteilt, die wiederum in 48 Genotypen unterteilt sind. Menschen können mit Noroviren der Genogruppen GI, GII, GIV, GVIII und GIX infiziert werden. Die anderen Norovirus-Genogruppen infizieren Nutztiere (z. B. Kühe und Schweine), Haustiere (z. B. Hunde und Katzen) und Wildtiere (z. B. Mäuse, Schweinswale und Fledermäuse). Trotz dieser großen Vielfalt an Noroviren, sind die Viren des Genotyps GII.4 die am häufigsten vorkommenden Noroviren in Menschen. Wie bei den Influenzaviren entwickeln sich die GII.4-Noroviren schrittweise weiter, wobei alte Varianten durch Neue ersetzt werden. In welchen Wirten sich diese Viren zu einer neuen Variante entwickeln, ist nicht bekannt. Einige der Nicht-GII.4-Noroviren werden nur selten gefunden, und es ist unklar, ob diese Viren nur in Menschen zirkulieren oder ob sie in den Perioden, in denen sie nicht in der menschlichen Population nachgewiesen werden, auch in anderen Wirtsreservoiren zirkulieren. In dieser Arbeit haben wir die Rolle von Tieren und Menschen bei der Entwicklung und Verbreitung der Noroviren untersucht.

Verbreitung der Noroviren an der Schnittstelle von Mensch und Tier

In **Kapitel 2** haben wir uns der Frage gewidmet ob Tiere eine Rolle in der Verbreitung von Noroviren spielen. Mehrere Studien haben dies untersucht, und wir haben diese Ergebnisse in **Kapitel 2.1** zusammengefasst. Wir konnten kaum Hinweise finden, die darauf hindeuten, dass tierische Noroviren Menschen auf natürliche Weise infizieren. Im Gegensatz dazu, können mindestens sieben Tierarten experimentell mit humanen Noroviren infiziert werden und in mehreren Studien wurde genetisches Material von humanen Noroviren in Tierkot nachgewiesen. In **Kapitel 2.2** haben wir die bei Tieren gefundenen humanen Noroviren mit den in Menschen zirkulierenden Viren verglichen. Anhand dieses Vergleichs können wir abschätzen, wie lange diese Viren bereits in Tieren zirkulieren. Wir wissen, dass genetische Veränderungen, die zu Änderungen des VP1-Proteins (vor allem der P Domäne) führen, die Eigenschaften des Virus beeinflussen können, da die Struktur dieses Proteins bestimmt, wie das Virus an die Zelloberfläche des Wirts bindet, und es auch der Teil des Virus ist, der vom Immunsystem des Wirts erkannt wird. Veränderungen dieses Proteins können deshalb sowohl die Art und Weise verändern, wie das Virus die Wirtszelle erkennt, als auch zu einer Immunumgehung führen, was bedeutet, dass Noroviren vom Immunsystem des Wirts nicht mehr erkannt und eliminiert werden können.

Bei der Untersuchung der genetischen Merkmale menschlicher Noroviren in Tieren fanden wir Unterschiede zwischen GII.7 und GII.17 die in Makaken gefunden wurden und GII.7 und GII.17 die in Menschen zirkulieren. Es ist möglich, dass sich diese Viren an ihre tierischen Wirte angepasst haben. Die Ansammlung von Mutationen (Veränderungen im genetischen Material) deutet darauf hin, dass diese Viren unbemerkt in Tieren oder Menschen zirkulierten.

Jede Infektion beginnt mit der Bindung des Virus an die Wirtszelle. In Folge gelangt das Virus in die Zelle und initiiert den Replikationszyklus, welcher dann zur Freisetzung von Viruspartikeln führt, die wiederum neue Zellen infizieren können. Welche Moleküle auf den Wirtszellen erkannt werden, ist abhängig von dem jeweiligen Virus. Noroviren binden an eine Gruppe von Zuckern, die als Blutgruppenantigene (Histo-bloodgroup antigens, HBGAs) bezeichnet werden. Diese befinden sich auf der Oberfläche von Darmzellen und werden auch im Speichel ausgeschieden. Beim Menschen hat sich gezeigt, dass die Bindung von Noroviren an Speichel oder Gewebe des Magen-Darm-Trakts (den Noroviren infizieren) mit der Anfälligkeit einer Person für Noroviren korreliert. Um zu testen, welche Tierarten potenziell mit menschlichen Noroviren infiziert werden können, haben wir die Bindungsspezifität von Noroviren an das Darmgewebe verschiedener Tierarten untersucht (**Kapitel 2.3**). Wir fanden heraus, dass mehrere humane Norovirus-Genotypen an das Darmgewebe von Schweinen, Hunden, Zwergfledermäusen, Schimpansen und Wildenten binden können. Im Gegensatz dazu binden weniger Genotypen an Darmgewebe von Katzen, Ratten, Schweinswalen, Puten, Hühnern und Austern. Die einzigen beiden Darmgewebe, an die keiner der humanen Norovirus-Genotypen gebunden hat, waren die der Lachmöwen und der Palmenflughunde.

Es gibt viele Arten von HBGAs und deren Verteilung ist von Person zu Person unterschiedlich. Aufgrund dieser Unterschiede sind einige Personen gegen eine Infektion mit bestimmten Norovirus-Genotypen resistent. Da dieser Zusammenhang bei Tieren noch nicht untersucht wurde, haben wir geprüft, ob die Bindung von Noroviren an tierisches Gewebe mit deren HBGA Typen und Verteilung zusammenhängt. HBGAs wurden in den Darmgeweben der meisten Tierarten und Individuen detektiert, aber wir fanden keine direkte Korrelation zwischen einem bestimmten HBGA Typen und der Bindung eines spezifischen Norovirus-Genotyps. Viren der GII Genogruppe banden an weniger Gewebe als Viren der GI Genogruppe, was darauf hindeutet, dass die GII Viren an weniger HBGA Typen binden können als GI Viren. Aufgrund des breiten Spektrums an Tierarten, die HBGAs im Gewebe des Magen-Darm-Trakts aufweisen, könnte das tierische Wirtsspektrum der humanen Noroviren sehr groß sein. Es ist jedoch wahrscheinlich, dass zusätzliche Faktoren die Anfälligkeit für Norovirus-Infektionen beeinflussen.

Die Rolle des Menschen in der Entwicklung und Verbreitung von Noroviren

In **Kapitel 3** haben wir uns mit der Rolle des Menschen in der Ausbreitung und Evolution von Noroviren befasst. Die mit Noroviren verbundene Krankheits- und Todeslast ist am höchsten bei Kindern unter 5 Jahren und Menschen über 65 Jahren. Da eine hohe Diversität an Noroviren in Kindern gefunden wurde, wurde die Hypothese aufgestellt, dass bestimmte Norovirus-Genotypen, die nur sporadisch gefunden werden, bei Kindern unerkannt zirkulieren. In **Kapitel 3.1** haben wir diese Hypothese mittels einer Analyse des Antikörperrepertoires von Kindern im Alter von 0-5 Jahren untersucht. Jede Infektion, die das Immunsystem aktiviert, führt zur Bildung von Antikörpern gegen den infektiösen Erreger. Diese Erreger-spezifischen Antikörper können noch Monate bis Jahre nach der Infektion im Blut eines Menschen nachgewiesen werden. Die Charakterisierung der im Blut vorhandenen Antikörper wird daher genutzt, um durchgemachte Infektionen einer Person zu identifizieren. Mit Hilfe eines Protein-Mikroarrays haben wir Antikörper in Seren von ~300 Kindern gegen die VP1 P Domäne von ~30 humanen Norovirus-Genotypen getestet. Wir untersuchten sowohl Immunglobulin G (IgG), den im Blut am häufigsten vorkommenden Antikörpertyp, als auch Immunglobulin A (IgA), das in geringerer Konzentration im Blut, aber am häufigsten in Sekreten wie Speichel und Schleim vorkommt. Über 90% der Seren der jüngsten Kinder (0-6 Monate) wiesen IgG auf, die mindestens einen Norovirus-Genotyp erkannten. Dabei handelt es sich wahrscheinlich um mütterliche Antikörper, die während der Schwangerschaft die Plazenta passieren und das Neugeborene das erste halbe Jahr vor Infektionen schützen. In den Seren von Kindern, die älter als 6 Monate waren sank der Spiegel der Norovirus-spezifischen IgG Antikörper, was auf einen Rückgang der mütterlichen Antikörper hindeutet. Bei den mütterlichen Antikörpern handelt es sich hauptsächlich um IgG. Daher fanden wir bei Kindern unter 6 Monaten nur wenige Seren mit Norovirus-spezifischen IgA. In Seren älterer Kinder (>1 Jahr) stieg der IgA-Spiegel an und war bei den ältesten Kindern (5.5 Jahre) am höchsten. Dies deutet darauf hin, dass Kinder in den ersten Lebensjahren bereits eine erste Norovirus-Infektion durchleben.

Viele Seren enthielten IgG, die mehr als einen Genotyp erkannten, wahrscheinlich aufgrund von Kreuz-Reaktivität. Dies bedeutet, dass Antikörper verschiedene Genotypen erkennen, die Ähnlichkeiten in Regionen aufweisen, die von Antikörpern erkannt werden. Es war daher unmöglich, festzustellen, mit welchen Genotypen diese Kinder zuvor infiziert worden waren. Um dies zu umgehen, analysierten wir als nächstes ausschließlich Seren, die ein signifikant höheres Signal gegen einen einzigen Genotyp zeigten. Diese Antikörper erkannten Genotypen, die am häufigsten Norovirus-Ausbrüche verursachen. Dies bedeutet, dass die standardmäßig durchgeführte Überwachung der Norovirus-Verbreitung in Kindern repräsentativ ist.

Eine weitere Gruppe, die für die Entwicklung und Ausbreitung von Noroviren wichtig sein könnte, sind Menschen mit einer Immunschwäche. Bei gesunden Menschen dauern akute Norovirus-Infektionen nur wenige Tage an, und das Virus wird oft nicht länger als zwei Wochen ausgeschieden. Bei Immungeschwächten können Norovirus-Infektionen hingegen chronisch werden und somit jahrelang andauern, wobei die Patienten Noroviren kontinuierlich im Stuhl ausscheiden. Während dieser langwierigen Infektionen häufen sich Mutationen in den Noroviren an, wodurch sie sich von Noroviren, die in gesunden Menschen zirkulieren, unterscheiden. Es gibt noch keine zugelassene Behandlung für diese Patienten, aber eine experimentelle Behandlung, die sich in mehreren Fällen als erfolgreich erwiesen hat, ist die orale Verabreichung von Immunglobulin (Ig)-Präparaten. Diese Präparate enthalten Antikörper aus dem Blut von mehr als 1000 Erwachsenen und beinhalten damit das durchschnittliche Antikörperspektrum der erwachsenen Bevölkerung. Ig-Präparate erkennen und binden an das VP1-Protein und blockieren so die Bindung des Virus an die HBGAs auf den Darmzellen, wodurch eine Infektion verhindert wird. Bei einigen Patienten führte diese Behandlung zu einer erfolgreichen Beseitigung der Norovirus-Infektion, bei anderen jedoch nicht. In **Kapitel 3.2** haben wir einen möglichen Grund für diese Erfolgsunterschiede der Ig-Behandlung untersucht. Zu diesem Zweck haben wir die genetischen Merkmale der Noroviren bei chronisch infizierten Patienten bestimmt. Im Laufe mehrerer Monate häuften sich Mutationen an, insbesondere in der P Domäne des VP1-Proteins, die für die Norovirusbindung und die Erkennung durch das Immunsystem wichtig ist. Bei einigen Patienten konnte die Bindung dieser Viren durch die Antikörper in der Ig-Behandlung nicht mehr blockiert werden, was wahrscheinlich die Wirkung der Behandlung verringert. Dies könnte erklären, warum einige Patienten nach der Behandlung nicht geheilt sind, und deutet darauf hin, dass die Erfolgsaussichten größer sind, wenn Ig in einem frühen Stadium der Infektion verabreicht wird.

Die Rolle der Polymerase bei der Entwicklung und Verbreitung von Noroviren

In **Kapitel 4** haben wir einen möglichen Mechanismus untersucht, der die Verbreitung und Prävalenz neuer Noroviren beeinflussen könnte. Wenn eine Person gleichzeitig mit zwei verschiedenen Noroviren infiziert ist, kann genetisches Material zwischen diesen Viren ausgetauscht werden (Rekombination). Für Noroviren wurde nachgewiesen, dass Rekombination zwischen dem offenen Leserahmen 2 (open reading frame, ORF), der für das VP1-Protein kodiert, und dem ORF1, der für die Nichtstruktur Proteine kodiert, stattfindet. Diese Nichtstrukturproteine sind nicht Teil des Viruspartikels, spielen aber während des Infektionszyklus in der Zelle eine wichtige Rolle.

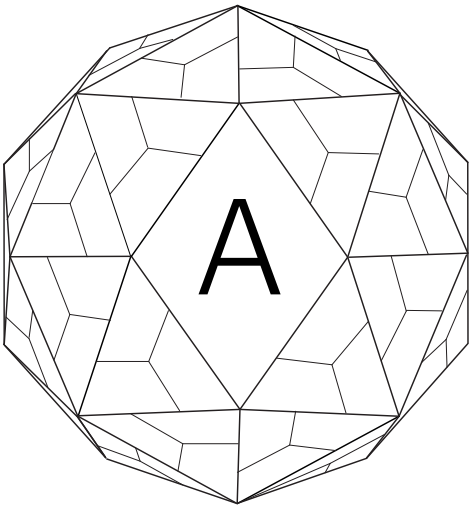
Humane Noroviren haben sechs Nichtstrukturproteine, eines davon ist die RNA-abhängige RNA-Polymerase (RdRp). Die RdRp ist das Enzym, das für die Replikation des viralen Genoms in der Zelle wichtig ist. Eine höhere RdRp-Aktivität

könnte dazu führen, dass in einem Wirt mehr Viren produziert werden und dass folglich mehr Viren ausgeschieden werden, wodurch sich die Wahrscheinlichkeit einer Ausbreitung auf einen anderen Wirt erhöhen kann. Wir verglichen die RdRp-Aktivität verschiedener Noroviren und obwohl sich die RdRps in ihrer Aktivität unterschieden, war dies nicht aussagekräftig dafür, wie häufig ein bestimmtes Norovirus bei Ausbrüchen gemeldet wird. Einige RdRp-VP1-Kombinationen werden besonders häufig bei Ausbrüchen gemeldet, und bei der Messung der RdRp-Aktivität in Anwesenheit verschiedener VP1-Genotypen konnten wir feststellen, dass einige Kombinationen zu einer höheren RdRp-Aktivität führten als andere. Dieser Effekt war nicht Genotyp-spezifisch und RdRp-VP1-Kombinationen, die häufig vorkommen, führten nicht unbedingt zu einer höheren RdRp-Aktivität als Kombinationen, die weniger häufig vorkommen. Daraus schlossen wir, dass die RdRp möglicherweise eine Rolle bei der Ausbreitung von Noroviren spielt, aber die genaue Rolle ist noch unklar.

Schlussfolgerung

Die Norovirus-Forschung hängt von molekularen Systemen und Techniken ab, und in **Kapitel 5** haben wir die derzeit verfügbaren Techniken beschrieben, die gebraucht werden, um unser Verständnis der Norovirus-Übertragung von Wirt zu Wirt zu verbessern. Zellkulturmodelle sind von entscheidender Bedeutung für die Charakterisierung und den Vergleich verschiedener Noroviren im Hinblick auf die Replikationskinetik und die Virus-Wirt-Interaktionen. Des Weiteren sind Tiermodelle wertvoll für die Untersuchung von Zelltropismus, Pathologie und Virus-Übertragung.

Die in dieser Arbeit beschriebenen Forschungsarbeiten tragen zum aktuellen Wissensstand über potenzielle Reservoire zirkulierender Noroviren bei. Die vorgestellten Ergebnisse deuten darauf hin, dass auch Tiere und Immungeschwächte in dieser Hinsicht eine Rolle spielen könnten, daher sollten beide weiter untersucht werden. Darüber hinaus sollte die Norovirus-Überwachung auch Populationen einbeziehen, die derzeit von der standardmäßig durchgeführten Überwachung nicht erfasst werden, wie z. B. verschiedene Tierarten und asymptomatische Norovirus-infizierte Personen. Zu diesem Zweck könnte der in dieser Arbeit beschriebene Protein-Mikroarray zur Messung der Exposition gegenüber menschlichen Noroviren verwendet werden. Wir beschreiben auch, dass die Polymerase zur schnellen Ausbreitung einiger Noroviren beitragen könnte, aber nicht zu der Ausbreitung anderer. Obwohl das VP1-Protein bisher von den Norovirus-Proteinen am ausführlichsten untersucht wurde, sind wir der Meinung, dass die Rolle der Nichtstrukturproteine bei dem Auftreten des Virus weiter untersucht werden sollte. Letztendlich ist das Verständnis des Reservoirs und der molekularen Mechanismen, die dem Auftreten von Noroviren zugrunde liegen, von entscheidender Bedeutung, um angemessen in die Ausbreitung von Noroviren eingreifen zu können.



Appendix

PhD portfolio
Curriculum vitae
Acknowledgments

PhD Portfolio

Name	Nele Villabruna
Research department	Viroscience, Erasmus MC
Research school	Post-graduate Molecular Medicine (MolMed)
PhD period	2016-2021
Promotor	Prof. Dr. Marion P.G. Koopmans
Co-promotor	Dr. Miranda de Graaf

Education

2016-2021	**PhD program** Department of Viroscience, ErasmusMC, Rotterdam, the Netherlands
2013-2014	**Master in Applied Marine Science** University of Plymouth, UK
2009-2013	**Bachelor of Science in Biology** University of Basel, Switzerland

PhD training

Courses		**Workload**
2019	Biomedical English Writing and Communication	3.0 ECTs
2018	Course in Virology	1.4 ECTs
2018	Workshop Presenting Skills for junior researchers	1.0 ECTs
2017	Virus Pathogen Resource Workshop	0.3 ECTs
2017	Course on Molecular Medicine	0.6 ECTs
2017	Basic Introduction Course on SPSS	1.0 ECTs
2017	Blogging for Scientists	0.3 ECTs
2016	Basic course on R	1.4 ECTs
2016	Workshop on GraphPad Prism 6	0.3 ECTs
2016	Workshop on Photoshop and Illustrator	0.3 ECTs
2016	Research integrity	0.3 ECTs
2016	SCIENION's two-day user course	0.6 ECTs

Seminars and Workshops

2018	Satellite symposium (ASV 2018): The Impact of the Microbiome on Virus Infection	3.0 ECTs
2018	Young Researchers Career Day: Workshop personal leadership & 150-word pitch	0.3 ECTs
2018	I&I Science Symposium	0.3 ECTs
2017	KNAW Masterclass: One Health Approach to Infectious Diseases	0.6 ECTs
2017	Satellite symposium (ASV 2017) Ecology and Evolution	0.3 ECTs

Oral & Poster presentations

2020	The 39th annual meeting of the American Society for Virology (ASV), USA Flash talk & poster, meeting canceled due to COVID-19 pandemic
2019	The 7th international Calicivirus Conference, Australia Presentation: **The pattern of norovirus attachment varies among species**
2019	General meeting of the Netherlands center for one health (NCOH), the Netherlands Poster & Pitch: **Can norovirus jump the species barrier?**
2019	The 7th European Congress of Virology (ECV), the Netherlands Poster: **Norovirus attachment to intestinal tissue of different species**
2019	Young COMPARE meeting, Denmark Presentation & poster: **Norovirus attachment to intestinal tissue of different species**
2019	The 23th Molecular Medicine day, the Netherlands Poster: **Norovirus attachment to intestinal tissue of different species**
2018	The 37th annual meeting of the American Society for Virology (ASV), USA Presentation: **Profiling of antibody responses to structural and non-structural proteins after norovirus infection**
2018	Young COMPARE, Denmark Presentation & poster: **Serological responses to norovirus structural and non-structural proteins after infection**
2018	The 22th Molecular Medicine day, the Netherlands Poster: **Profiling of antibody responses to structural and non-structural proteins after norovirus infection**
2017	Young COMPARE, the Netherlands Presentation & Poster: **Serological responses to norovirus structural and non-structural proteins after infection**
2017	The 21th Molecular Medicine day, the Netherlands Poster: **Serological responses to norovirus structural and non-structural proteins after infection**
2016	Young Antigone meeting, UK Presentation: **Serological responses to norovirus structural and non-structural proteins after infection**

Teaching

9/2017-6/2018	Supervision of a HBO student

List of Publications

Villabruna N, Izquierdo-Lara RW, Schapendonk CME, de Bruin E, Chandler F, Thao TTN, Westerhuis BM, van Beek J, Kohns Vasconcelos M, Sigfrid L, Giaquinto C, Goossens H, Bielicki JA, Fraaij PLA, Koopmans MPG and de Graaf M.
Profiling of humoral immune responses to norovirus in children across Europe.
Submitted

Izquierdo-Lara RW, **Villabruna N**, Hesselink DA, Schapendonk CME, Meier JIJ, Goodfellow IG, Dalm VASH, Fraaij PLA, Van Kampen JJA, Koopmans MPG and de Graaf M.
Intrahost multi-lineage evolution of human norovirus in immunocompromised individuals: implications for oral immunoglobulin treatment.
Manuscript in preparation

van Kampen JJA, Dalm VASH, Fraaij PLA, Oude Munnink BB, Schapendonk CME, Izquierdo-Lara RW, **Villabruna N**, Ettayebi K, Estes MK, Koopmans MPG and de Graaf M.
Clinical and in-vitro evidence favoring immunoglobulin treatment of chronic norovirus infection in a patient with common variable immunodeficiency
Submitted

Villabruna N, Schapendonk CME, Aron GI, Koopmans MPG and de Graaf, M. (2021). **Human noroviruses attach to intestinal tissues of a broad range of animal species**. Journal of Virology, 95(3), e01492-20.

Villabruna N, Izquierdo-Lara RW, Szarvas J, Koopmans MPG and de Graaf M. (2020). **Phylogenetic investigation of norovirus transmission between humans and animals.** Viruses, 12(11), 1287.

Villabruna N, Koopmans MPG and de Graaf, M. (2019).
Animals as reservoir for human norovirus. Viruses, 11(5), 478.

De Graaf M, **Villabruna N** and Koopmans MPG. (2017).
Capturing norovirus transmission. Current Opinion in Virology, 22, 64-70.

Curriculum vitae

Nele Villabruna was born on the 17th of August 1988 in Basel, Switzerland. After completing the Gymnasium in 2008, she studied Biology at the University of Basel, Switzerland. During this time, she became interested in Marine Biology and therefore, after receiving her Bachelor's degree in 2013, Nele continued to study Applied Marine Sciences at the University of Plymouth, United Kingdom. For her Master's thesis, she focused on the giant *Emiliana huxleyi virus*, which infects *Emiliania huxleyi*, one of the most abundant algae species. Under the supervision of Prof. Mike Allen, she characterized the viral protein EHV298 and its interactions with various virus and host factors. Working on these fascinating viruses sparked Nele's interest in virology and after receiving her Master's degree in 2014, she changed her focus from giant marine viruses to human viruses and worked as a research assistant at the Heidelberg University in Germany. In the arbovirus group, led by Dr. Pierre-Yves Lozach, she studied cell entry of the tick-borne *Uukuniemi virus*. In 2016, she started her PhD under the supervision of Dr. Miranda de Graaf and Prof. Dr. Marion Koopmans in the department of Viroscience, Erasmus MC, Rotterdam, the Netherlands. During her PhD trajectory, Nele investigated noroviruses at the human-animal interface and the role of different hosts in norovirus circulation as well as the emergence of novel variants and recombinants.

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