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Capturing norovirus transmission

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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.

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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 [1]. 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 [2–4]. 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 [5,6**,7–9].

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 [10]. 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 [11–13]. 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 (Figure 1) [14]. 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. 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 [15,16]. 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 [17–19]. Persons can be infected with HuNV without the presentation of symptoms [20], but the relationship between shedding and disease is not clear with some conflicting evidence in literature [4,21].

However, in health-care settings, symptomatic patients were found to be responsible for the majority of transmission events [22]. In all, epidemiological studies have also provided some information on differences in transmission efficiency between genotypes, but it is difficult to

Figure 1
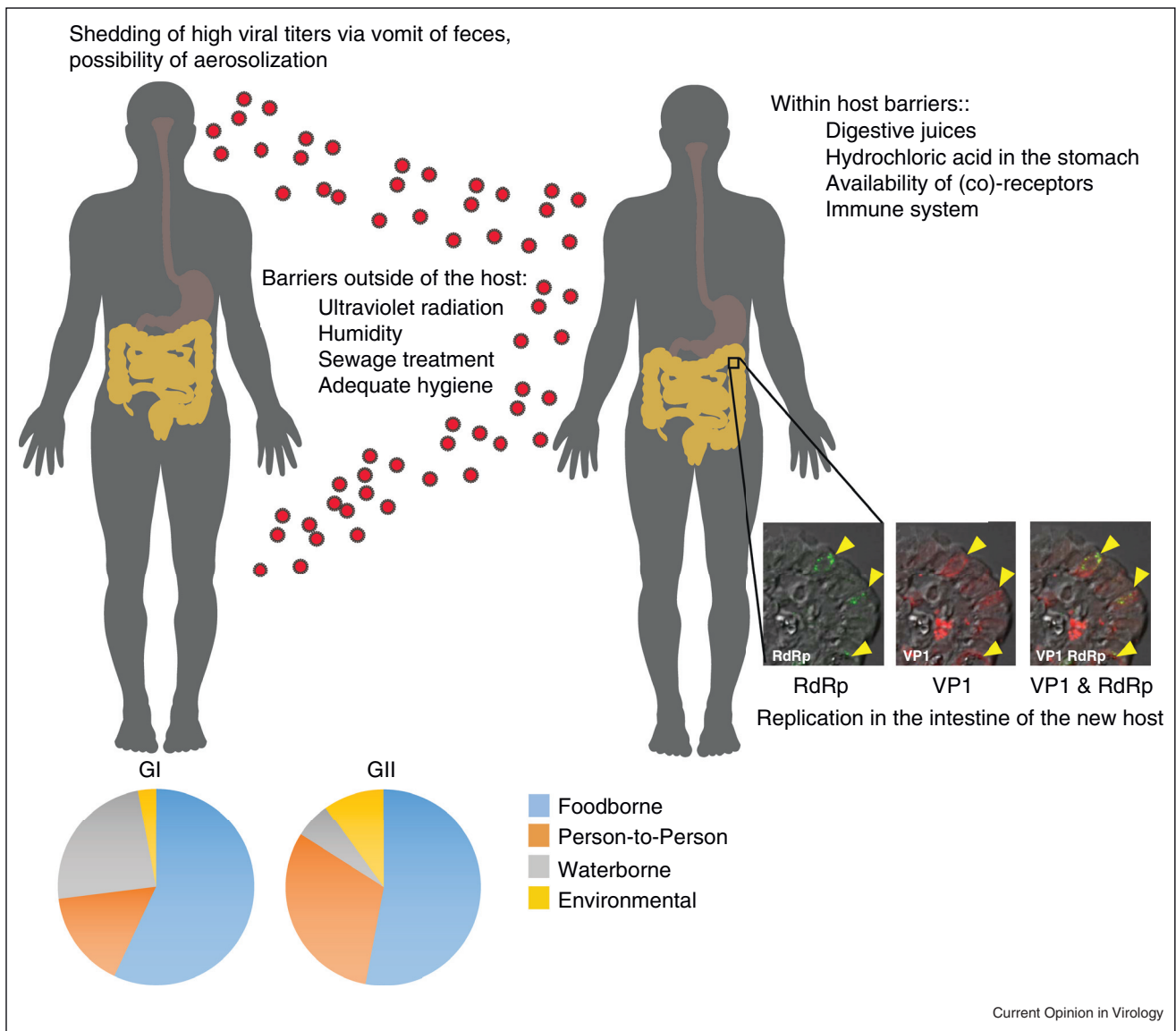
HuNV tropism and transmission. After shedding from the host *via* vomitus or feces HuNV is transmitted to the next host. Transmission can occur through several routes, with differences in association between genogroup and transmission route [68]. During transmission, viruses encounter multiple environmental barriers and as well as structural, functional and immunological barriers within the host 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 [25]. The inset shows the detection of RdRp and VP1 (yellow arrows) in the same duodenal biopsy from a HuNV positive patient (adapted from [25]).

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 [23,24••]. 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 detection of the non-structural proteins RNA-dependent RNA polymerase (RdRp) and the genome associated VPg. Both were detected alongside VP1 in duodenal and jejunal enterocytes [25••] (Figure 1). In agreement with these findings successful cultivation of multiple HuNV strains in human intestinal enteroid

monolayers was recently reported [26••]. 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 [27]. 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 researches 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 [28]. However, these infection models are not suitable for co-culturing 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* [29].

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 [30–34]. Most of these animal models can be infected with HuNV by oral inoculation. Despite the apparent stability of NV in an acidic environment [35], 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 [32].
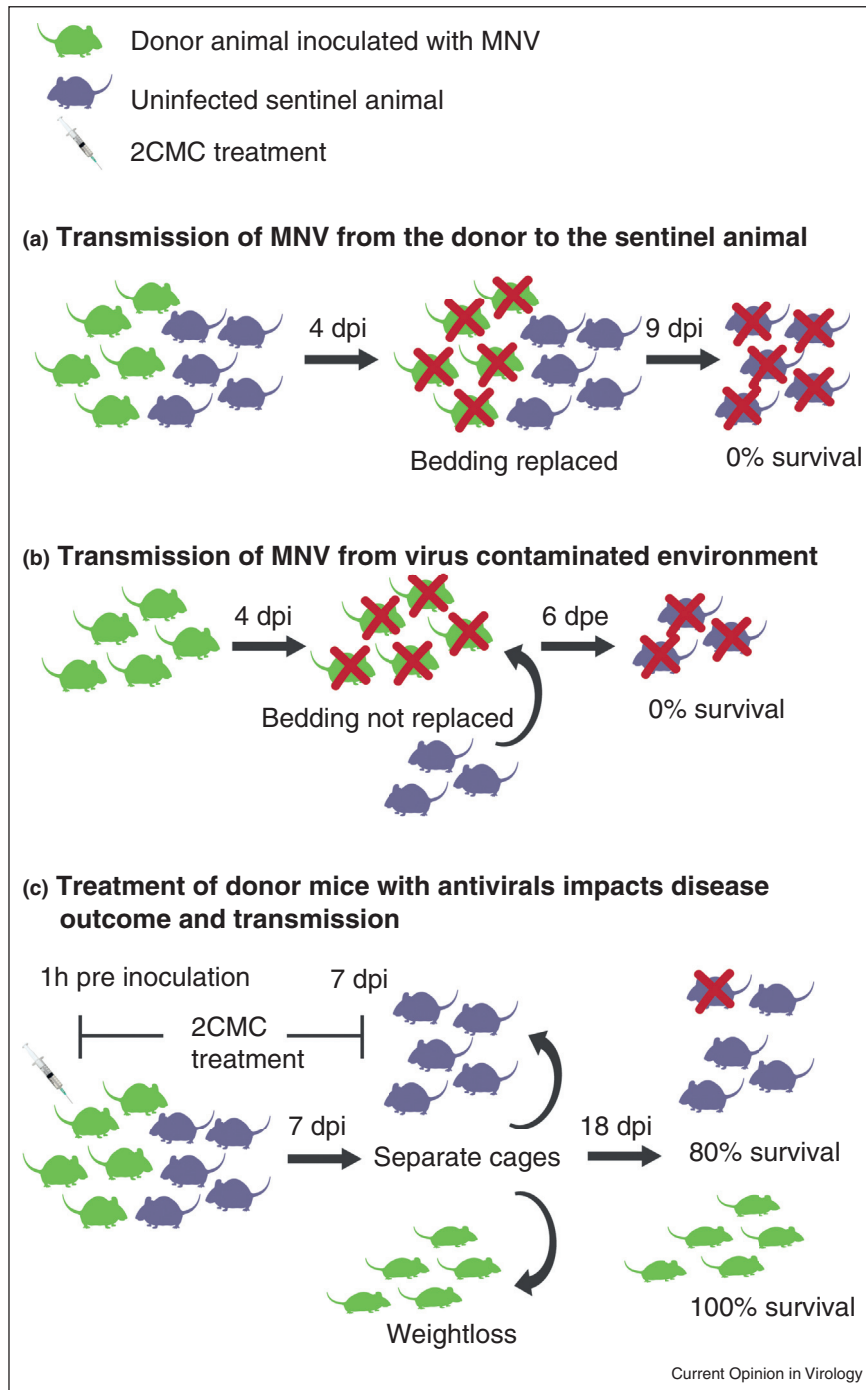
Pigs are natural hosts for NV genotypes GII.11, GII.18 and GII.19 [10,36,37], while bovine species are natural hosts for GIII strains [38]. One study reports the detection of GII.4 in pigs and cattle from farms [37] and inoculation of both gnotobiotic pigs and calves with GII.4 results in replication [31]. 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 [39]. Contaminated oysters can be a source of foodborne HuNV infection in humans and this can be mimicked in gnotobiotic pigs as they can be infected by feeding them HuNV

seeded oyster homogenates [40]. 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 [41]. 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 [42]. 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 [43,44]. 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 [45], co-inoculation with *Enterobacter cloacae* inhibited HuNV infectivity in pigs [46].

Chimpanzees can be infected with GI.1 HuNV by the intravenous and oral route [33,34]. 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 [34]. The virus could be passaged from chimpanzee-to-chimpanzee by feeding of fecal filtrate [33]. 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 [33]. 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 [47,48]. 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 [49]. MNV belongs to genogroup GV and replicates to high titers *in vitro* and *in vivo* in its natural host [49,50]. 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 [51]. 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 [51]. These studies can also be performed with HuNV; the degradation of virus particles can be assessed by determining the change in viral RNA copies

Figure 2

Legend:
- Donor animal inoculated with MNV
- Uninfected sentinel animal
- 2CMC treatment

(a) **Transmission of MNV from the donor to the sentinel animal**

4 dpi → Bedding replaced → 9 dpi → 0% survival

(b) **Transmission of MNV from virus contaminated environment**

4 dpi → Bedding not replaced → 6 dpe → 0% survival

(c) **Treatment of donor mice with antivirals impacts disease outcome and transmission**

1h pre inoculation — 2CMC treatment — 7 dpi

7 dpi → Separate cages → 18 dpi → 80% survival

Weightloss → 100% survival

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Mice model for contact transmission Mice, deficient in INF-α/β and IFN-γ receptors were infected with MNV (green). Uninfected sentinel mice (purple) were placed with the infected donor mice (a) or in a contaminated environment in absence of the infected donor animals (b), both settings resulted in infection of the sentinel mice. 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 (c). The 2CMC treatment impacted on disease and transmission [53].

or binding properties [52], but such assays do not always accurately represent infectious titers. A transmission model for MNV was developed by Rocha-Pereia et al. [53••] (Figure 2). 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 (Figure 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 (Figure 2c). Thus, the mice 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.

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 [54], 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 [55]. 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 affect 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 [56•]. 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 [57]. 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 [56]. Others were able to generate aerosols with the HuNV surrogate bacteriophage MS2 [58]. 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 [59]. Considering the low infectious dose needed to infect volunteers [3], 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 [45,60]. In the presence of bacteria MNV was more stable to electrical breakdown in water [61•], 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* [62]. High humidity also resulted in a decrease of infectivity and binding capacity of MNV and HuNV, respectively [63], 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 [64]. Despite its sensitivity to humidity, HuNV is very stable in water. It was demonstrated that ground water spiked to a final concentration of $\sim 6.5 \times 10^7$ 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 virus [65]. 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 [8,66], and limited removal efficiency during sewage treatment [67].

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.

Acknowledgements

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