1 Norovirus genetic diversity and evolution: implications for antiviral
2 therapy

3 *Joana Rocha-Pereira, Jana Van Dycke and Johan Neyts**

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6 KU Leuven– University of Leuven, Department of Microbiology and Immunology, Rega
7 Institute for Medical Research, Laboratory of Virology and Chemotherapy, Leuven,
8 Belgium

9 *Corresponding author. E-mail address: Johan.Neyts@kuleuven.be. Rega Institute for
10 Medical Research, KU Leuven, Minderbroedersstraat 10, B-3000 Leuven, Belgium.

14 ABSTRACT

15 Human noroviruses are the leading cause of foodborne illness causing both acute and
16 chronic gastroenteritis. In recent years, a number of vaccine candidates entered (pre-)
17 clinical development and the first efforts to develop antiviral therapy have been made.
18 We here discuss aspects of norovirus genetic evolution, persistence in
19 immunocompromised patients as well as the risk and potential consequences of
20 resistance development towards future antiviral drugs.

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Norovirus: clinical relevance, spectrum of disease and the need for therapy

Human noroviruses are the most common cause of foodborne illness resulting in both acute and chronic gastroenteritis. Even though the majority of norovirus infections are cleared within 2-3 days, vulnerable populations (the elderly, young children, immunocompromised) often present symptoms for longer periods of time for which hospitalization may be needed. Annually an estimated 212.000 die due to a norovirus infection [1]. Common short-duration norovirus infections repeatedly lead to outbreaks affecting hundreds of individuals in confined locations such as hospital wards, schools, military facilities, cruise ships, hotels etc. which illustrates the type of societal disruption caused by these viruses. The total societal costs of these infections have recently been estimated at \$60 billion /year and \$4 billion in direct health system costs [2]. There are an estimated 684 million infections every year, spread through all age groups across the globe [1,3].

Norovirus infections are increasingly referenced in transplantation and oncology hospital units [4]. Although more comprehensive studies are necessary to fully understand the size of the problem, the prevalence of norovirus-caused diarrhea was reported to be ~18% in solid organ transplant (SOT) and hematopoietic stem-cell transplant (HST) recipients, with the infection lasting 6 months on average [5-8]. The notion that these can become more and more debilitating and life-threatening over time has raised in recent years, as has the number of reported cases of chronic gastroenteritis. The impact of outbreaks in such circumstances has been reviewed in [4].

Norovirus outbreaks are extremely difficult to control since the virus spreads from person-to-person *via* aerosolized virus particles of vomitus or stool of infected individuals [9], it

has an infectious dose of ~18 virus particles, can persist infectious on surfaces for up to 2 weeks and is resistant to most common disinfectants [10-12]. Hospital norovirus outbreaks are highly disruptive events. Public Health England, through the Hospital Norovirus outbreak Reporting Scheme (HNORS) reported close to 1000 laboratory-confirmed norovirus hospital outbreaks per year in England and Wales (2009-2015), which affected more than 70 000 patients, 18 000 staff and led to 52 000 days of ward closure.

Despite the ample need to control and prevent norovirus infections and outbreaks, no vaccine or specific antiviral drug is currently available. Supportive therapy (electrolyte replenishment) is provided to dehydrated individuals, while reduction of immunosuppressive therapy is sometimes attempted in transplant recipients, in an effort to allow the immune response to clear the infection. Due to the pressing need for treatment, the effect of approved drugs such as ribavirin and nitazoxanide has been evaluated in a small number of patients but no clear beneficial clinical effect was observed [13-15].

Considerations on norovirus biology, genetic diversity and evolution

Genome organization

Noroviruses are non-enveloped single-stranded positive-sense RNA viruses which belong to the family of the *Caliciviridae*. The RNA genome of 7.4–7.7 kb is organized into three open reading frames (ORF1-3). The ORF1 of norovirus encodes the six/seven nonstructural proteins in the following order: the p48/ N-terminal protein (or NS1-2), the

NTPase (NS3), the p22 (NS4), the VPg (NS5), the viral protease (Pro, NS6), and the viral RNA-dependent RNA polymerase (RdRp, NS7). ORF2 and 3 encode the major and minor structural capsid proteins VP1 and VP2, respectively. The murine norovirus (MNV) has an additional ORF4 which encodes virulence factor 1 (VF1) [16,17]. Virions contain 180 copies or 90 dimers of VP1, the major structural protein, that assemble into icosahedral particles. The VP1 protein is divided into a conserved internal shell domain (S) and a more variable protruding domain, the P domain, which forms the arch-like protrusions. Its P2 subdomain is located at the outmost surface of the viral capsid and comprises a hypervariable region, where resides the binding interface for histo-blood group antigen (HBGA) association with norovirus [18]. The VP2 is a small basic structural protein which is present in one or two copies per virion and associates with the VP1 S domain at the interior surface of the capsid, being likely involved in the capsid assembly and genome encapsidation [19].

Genetic diversity and the potential for zoonotic transmission

Noroviruses are divided into six genogroups which have > 60% amino acid sequence identity in the major structural protein VP1, of which genogroup I (GI) is further divided into 9 genotypes and GII into 22 genotypes (genotypes share > 80% amino acid identity in VP1). The (human) Norwalk virus is the prototype of the genus and is designated GI.1. Human pathogens are found in GI, GII and (rarely) GIV, GIII comprises bovine and ovine strains and GV murine noroviruses. In addition, swine strains are classified into GII, feline into GIV and canine strains into GIV and GVI [20]. There is no clear evidence that these viruses could be capable of crossing the barrier of species (due to the genetic similarity

between human and animal noroviruses). However, transmission between different host species has occurred: human noroviruses have been detected in pet dogs, pigs, cattle and rhesus macaques [21-23]. Antibodies against bovine and canine strains have been found in humans, with higher prevalence in veterinarians [24,25]. Conversely, antibodies against human noroviruses were detected in dogs [26,27]. Experimentally, human noroviruses can infect (i) both gnotobiotic pigs and calves via the oral route and cause diarrhea, (ii) chimpanzees when injected intravenously resulting in asymptomatic shedding of virus in the stool with seroconversion and (iii) Rag/gamma chain-deficient (Rag- γ c^{-/-}) balb/c mice via the intraperitoneal route leading to a short-lived asymptomatic infection [28-31]. Human norovirus virus-like particles (VLPs) were shown to bind to the intestinal tissue of dogs, even though viral replication was not demonstrated [27]. These data point out that it is at least possible that some of the circulating or newly emerging norovirus strains could have been circulating in other species (Fig.1).

GII.4 versus non-GII.4 noroviruses: escaping herd immunity?

Of the many norovirus genotypes, GII.4 noroviruses are of special importance since these cause the vast majority of outbreaks. Furthermore, novel GII.4 variants have emerged every 2-3 years in the past decades, replacing the previously dominating strain and becoming globally predominant [11,32]. This pattern of epochal evolution with cyclic emergence of strains causing worldwide epidemics resembles that of influenza virus [33]. These pandemic GII.4 variants can escalate the number of outbreaks, are preferably transmitted person-to-person and affect disproportionately patients older than 65 years in health care settings [9]. On the contrary, non-GII.4 strains (GI.3, GI.6, GI.7, GII.3, GII.6,

and GII.12) are more likely to cause foodborne outbreaks [9]. Particularly GI viruses associate with waterborne outbreaks to a higher degree, which could be due to a greater stability of the GI viral capsid, when compared to GII [34].

The emergence of novel GII.4 strains has been linked to mutations that arise in the HBGA-binding P2 domain of VP1 [35,36], which result in antigenic changes making these viruses difficult to be recognized by antibodies generated against previously circulating strains. These findings imply that the virus is evolving either by broadening its capacity to bind cellular attachment factors and/ or receptors or by evading the immune system and escaping herd immunity (Fig.1). GII.4 noroviruses are indeed referenced as being able to bind a wider range of HBGAs than other genotypes [37], hence this can be an evolutionary advantage of these strains.

Homologous recombination

Homologous recombination is one important mechanism contributing to evolution of both GII.4 and non-GII.4 noroviruses . The ORF1-ORF2 junction is the region where this event occurs more frequently, but other sites such as the ORF2-ORF3 junction and within ORF2 have been identified [38,39]. The ORF1-ORF2 recombination facilitates the blend of different structural with non-structural portions of the genome, giving potentially rise to a virus with a more efficient polymerase, higher mutation rates, improved fitness and virulence. Inter- and intra-genotype recombination happens and it has impacted the emergence of GII.3, GII.4 strains (e.g. New Orleans 2009 and Sydney 2012) and potentially others [39,40]. Due to the increased recognition of this phenomenon, a revised

phylogenetic classification was set in place, where an ORF1/ RdRp genotype is attributed besides the standard capsid/ VP1 genotype [20]. Interestingly, some of these ORF1/ RdRp sequences are orphan, having no known capsid “match”. The respective genotypes are thus referenced with letters instead of numbers (e.g. GII.Pa), potentially until the identification of a matching capsid genotype. These viruses could be circulating within the human population without our knowledge or have originated in other species.

Multiple scenarios favor coinfections and therefore these recombination events (Fig.1), namely (i) foodborne outbreaks with multiple norovirus strains [41], (ii) chronic infections of immunocompromised hosts which present prolonged virus shedding with larger strain diversity (when compared to immunocompetent hosts) [11,42], (iii) norovirus infections of malnourished individuals, who present a greater number of mutations in the capsid together with increased severity of disease (as demonstrated in a mouse model [43]) .

Limitations to the success of recombination as condition towards evolution concern the replication strategy of norovirus. Some replication steps may act as bottlenecks, affecting the fitness and viability of recombinant strains. These include the interaction of the VPg (and the 5'-end of the genome) with the RdRp, the specificity of the cleavage sites of the protease, the enhancement of RdRp activity by its respective VP1, and moreover, the ability of the RdRp to discriminate its subgenomic substrate from that of another genogroup [44-46]. Hence, one may assume that intergenogroup recombination may be unlikely to happen or would require very special circumstances to originate viable replication-fit virions.

Reservoir and intra-host dynamics

One pressing question is what human or animal reservoirs exist for human noroviruses and which of these harbor the next pandemic strain (revised in [47]). The emergence of the GII.P17-GII.17 norovirus strain in 2014 [48], which became predominant in some parts of Asia but not yet in Europe and America is an intriguing event that will contribute to a better understanding of the norovirus evolution interplay. The acquisition of a novel ORF1 may have affected the replication rate of the virus or its ability to accumulate mutations. On the other hand, the interaction of the GII.17 capsid with HBGAs will condition the susceptible population (as will pre-existing immunity). Likely other antigenic and virulence determinants are present and also influence how the strain will circulate.

Whether norovirus-infected immunocompromised individuals are in fact the reservoir of new variants is a current hypothesis. In comparison to individuals with an acute infection and a normal immune system, the viral quasispecies appears to be more complex among the immunocompromised [49]. However, there is also less selective pressure of the immune system in these individuals. Intermediate immunity together with quasispecies diversity has been suggested to have the greatest potential for intra-host drift and evolution [47]. The elderly and malnourished children are populations with prolonged infection which may allow the larger accumulation of mutations and recombination events over the course of infection.

Host factors

Host factors such as age, health status and pre-existing immunity matter thus to the epidemiology of the different genotypes and condition the evolution of noroviruses. The role of HBGAs as attachment factors and/ or receptors has been extensively studied and revised [50-52]. The host fucosyltransferase (FUT) 1 2 and 3 genotype (ABO, Secretor, and Lewis genotype) determines their susceptibility to each norovirus strain. However, questions remain regarding the host cell tropism of human noroviruses, its entry process and ultimately how to robustly cultivate these viruses *in vitro*, despite recent advances [31,53]. A better understanding of the role of M cells, the Peyer's patches, the gut microbiome and other factors will bring significant progress and ultimately led us to more robust models and assays to study the biology and pathogenesis of noroviruses [54-56].

Studies of the immune response against human norovirus use thus VLP-HBGA blockade assays due to the unavailability of classic tissue culture neutralization assays. Such studies with human volunteers have been performed with multiple norovirus genotypes with variable outcomes, being likely affected by the pre-exposure history of volunteers [57]. Short-term immunity was demonstrated to be virus or serotype specific, whereas long-term immunity has been harder to put in evidence. The levels of intestinal secretory antibodies appeared to correlate with protection at a higher level than serum antibody titers, particularly in natural infections.

Antiviral strategies against a rapidly evolving virus

There are many target populations in need of a potent and safe norovirus antiviral therapy, and it may be challenging to find one solution that fits all the needs. These populations

include those with pre-existing disease and the risk for chronicity (transplant recipients, undergoing chemotherapy, immune deficiencies), specific age groups with increased risk for severe infection (young children, elderly), the occupationally exposed that can contribute to virus spread and thus outbreaks (food handlers, military, business travelers, health care workers) but also those engaging in leisure activities (such as restaurants, cruise ships, hotels, parties, sport events) within their family household or not. While in the first groups the emphasis can be on treatment of ongoing infections and reducing the severity of the disease, the key to control norovirus infections will likely be prophylaxis either by means of vaccines and/or small molecule antivirals.

Efforts towards vaccine development

Several vaccine candidates have been proposed for noroviruses, of which the most advanced is a GI.1/GII.4 bivalent VLP vaccine from Takeda (acquired from Ligocyte). Even though recent studies with two doses of this vaccine intramuscularly administered failed to show a significant reduction of illness upon re-challenge with a GII.4 norovirus [58], additional phase 2 studies are ongoing.

Herd immunity has been proposed to be the driving force of GII.4 noroviruses, since antigenic variation in the HBGA-binding P2 domain of VP1 has been linked to the emerging pandemic strains. Even though there are conserved features in the HBGA binding profiles (within the genotypes of each genogroup), noroviruses have a striking capacity to evolve by means of antigenic variations of the P domain. This scenario will very likely continue, with novel GII.4 strains emerging, perhaps GII.17 or other genotypes.

222 This will remain a challenge for vaccine development, hence efforts should be made to
223 anticipate such events. The design of a consensus GII.4 VLP has been a rational
224 approach [59], which could be extended to other genotypes/ genogroups; how successful
225 this will be depends on how broadly reactive the elicited antibodies will be [60]. Of great
226 importance is also to know whether other antibodies, against other capsid sites or viral
227 proteins play a role in the control of the infection and effective neutralization, potentially
228 targeting less variable domains [61,62]. One issue is the lack of an array of systems (and
229 assays) where quantification of antibody titers and neutralization becomes possible in a
230 sensitive and high-throughput manner.

231 Other important aspects to address and better define are what the important correlates
232 of protection are, to assess how immunogenic the vaccine is in the different populations
233 such as the elderly and young children and primarily to study the duration of the protective
234 immunity of such vaccine [63]. This could become a major challenge and directly
235 condition the applicability of a norovirus vaccine. Once such vaccine would become
236 available and widely used in certain target populations, it will constitute additional
237 “pressure” on the vast evolving noroviruses and could facilitate the emergence of new
238 strains, which could escape the vaccine (Fig.1).

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240 Antiviral therapy: treatment, prophylaxis, risk for development of drug resistance

241 The mentioned issues would apply to monoclonal antibodies, nanobodies but also small
242 molecule antivirals that target the viral capsid. The vast accumulation of mutations with
243 little or no fitness cost for the virus could lead to rapid resistance to this type of therapy

(Fig.1). A well-known example of this is the fast selection of resistance to the so called picornaviruses capsid binders, which hampers their clinical development.

Antivirals targeting non-structural proteins of noroviruses would have a greater chance of success. Particularly, targeting the viral enzymes of replication, i.e. the protease, the RdRp and the putative NTPase/helicase would bring two advantages. First, there is greater similarity between the enzymes of the different norovirus genogroups, both structurally and in terms of amino acid sequence, when compared to the capsid. The fact that one enterovirus protease inhibitor showed *in vitro* activity against a GI, GII and GV norovirus protease is illustrative of this [64]. Hence, molecules targeting the replication machinery have a greater chance of being active against noroviruses of every genogroup than compounds that target the viral capsid. Secondly, viruses have a much harder time in becoming resistant to such essential enzymes without losing fitness. A high-barrier to resistance is a well-known feature of inhibitors of the viral polymerase, particularly that of nucleoside analogues [65]. The effect of the nucleoside 2'-C-methylcytidine against GI (Norwalk replicon), GII.4 (infected B cells and Rag- γ C^{-/-} mice) and GV (infected RAW 264.7 macrophages and AG129 mice) noroviruses *in vitro* and *in vivo* further highlights the polymerase as a preferential target for treatment and prophylaxis of norovirus infections [66-70]. Furthermore, in a mouse norovirus model of persistent infection, multiple rounds of treatment with the nucleoside resulted in no phenotypic resistance [71]. More potent and safe newly-designed polymerase inhibitors that can completely suppress viral replication are highly desirable. An incomplete suppression of viral replication could lead not only to loss of drug sensitivity but also allow the select of mutations that confer higher replication rates to the viral polymerase [72,73]. Besides the RdRp, the protease

and the NTPase/helicase, norovirus encode other non-structural proteins (NS1-2 and NS4) and a VPg that all may be potential targets (either as such or the interaction with host cell factors) for inhibitors of norovirus replication [74,75]. Indeed, it remains largely unexplored what the molecular targets are for inhibition of norovirus replication.

Overall, a highly potent norovirus drug with a very safe profile may both be used prophylactically and therapeutically. When developing norovirus inhibitors one should aim to obtain (a) drug(s) that are not only pan-genotype active but that should also have a high barrier to resistance. Development of potential drug-resistance and the fitness of such variants, should be carefully monitored during clinical development. Possibly, at least two compounds, with non-overlapping resistance profiles may be developed that can be combined. In analogy to the fixed dose combination treatment for chronic infections caused by the hepatitis C virus and HIV, combining such inhibitors may allow to efficiently control noroviruses with a low risk of resistance development both in chronically infected patients and in an outbreak setting.

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Legend to the figure:

Figure 1 – Norovirus evolution is conditioned by the pressure imposed by future vaccines and antivirals (1) [once these become available] and by the host immune response (2). To escape the virus makes use of its human and potentially animal reservoirs (3), its quasispecies diversity and the efficiency and high mutation rate of the viral polymerase (4). Image of the norovirus was modified from the CDC library.

Fig. 1