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Norovirus genetic diversity and evolution: implications for antiviral therapy

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Human noroviruses are the leading cause of foodborne illness causing both acute and chronic gastroenteritis. In recent years, a number of vaccine candidates entered (pre-) clinical development and the first efforts to develop antiviral therapy have been made. We here discuss aspects of norovirus genetic evolution, persistence in immunocompromised patients as well as the risk and potential consequences of resistance development toward 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. Because of 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-γc^{-}/$^{-}$) 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 (Figure 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 disproportionally 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 (Figure 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-genotype 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]. Because of 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