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CHAPTER

STRUCTURE AND GENOTYPES OF NOROVIRUSES

4

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4.1 INTRODUCTION

Infection with human noroviruses is a leading cause of acute gastroenteritis affecting all age groups worldwide. According to a 2015 World Health Organization report on the estimates of global burden of foodborne diseases from 2007 to 2015, noroviruses and *Campylobacter* spp. are the top two causes (among biological and nonbiological agents) of foodborne illnesses. This highlights the public health importance of noroviruses with regard to the worldwide human population. Understanding the structure of noroviruses may provide important insights into why this group of enteric viruses is highly stable under hostile external environment and is very successful in person-to-person transmission. Noroviruses are genetically very diverse, and how this diversity influences public health and shapes the epidemiology are of clinical and research interest. Finally, as with most, if not all, viruses, noroviruses are continuously evolving, and it is not surprising that new variants of norovirus with better survival fitness occasionally emerge. What do we expect to see when a new norovirus variant emerges? Will a new variant reshape the current epidemiological landscape of norovirus gastroenteritis? In this chapter, we discuss the structure and genotypes of noroviruses as well as the latest research developments with an aim to provide more clues for those who are interested in pursuing the ultimate answers to these questions.

4.2 STRUCTURE

4.2.1 GENOME ORGANIZATION

Human noroviruses belong to the family *Caliciviridae* and the genus *Norovirus.* They have a relatively small, single-stranded, positive-sense, linear RNA genome approximately 7500 nucleotides in length. The first complete genome of Norwalk virus, the prototype (GI.1) of noroviruses, was deciphered in 1990 (Xi et al., 1990) (Fig. 4.1). The genome is organized into three overlapping open reading frames (ORF1−ORF3). ORF1, with a size of approximately 5100 bases, encodes for a polyprotein including structural protein VPg and nonstructural proteins such as 3C-like protease and 3D-like RNA-dependent RNA polymerase. ORF2, with a size of approximately 1600 bases, encodes for the major capsid protein called viral protein 1 (VP1), which is further subdivided into

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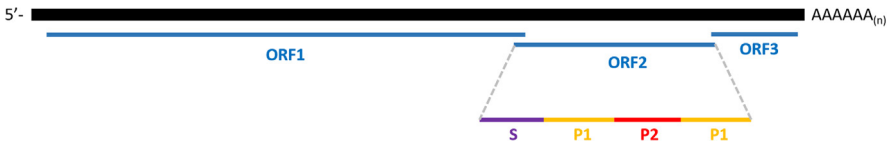

FIGURE 4.1

Genome organization of human noroviruses into three overlapping open reading frames (ORF1−ORF3). ORF1, -2, and -3 encode for polyprotein, major capsid protein VP1, and minor capsid protein viral protein 2, respectively. The diagram is not drawn in scale. P1, protruding domain 1; P2, protruding domain 2; S, shell.

the shell (S), protruding 1 (P1), and P2 subdomains (Fig. 4.1) (Prasad et al., 1999). ORF3, with a size of approximately 720 bases, encodes for the minor capsid protein called VP2, which may function to stabilize the capsid virion (Bertolotti-Ciarlet et al., 2003; Vongpunsawad et al., 2013). The 5′ end of the genome is covalently linked to VPg (Daughenbaugh et al., 2006), and the 3′ end contains a polyadenylated tail. Transfection of complete norovirus RNA genome can produce intact virus particles, suggesting that the genome itself is infectious (Guix et al., 2007).

4.2.2 VIRION STRUCTURE AND ENVIRONMENTAL STABILITY

Norovirus virion is nonenveloped and approximately 27 nm in diameter. Each virion is composed of 90 dimers of VP1 arranged on a T = 3 icosahedral symmetry with a cup-shaped morphology under electron microscopy (Chen et al., 2004; Dolin et al., 1982; Prasad et al., 1999). The P2 domain of VP1 is the most surface-exposed part of the virion and contains histo-blood group antigen binding interface (putative host attachment factor for noroviruses) and antigenic epitopes linked to immune escape (Cao et al., 2007; Chen et al., 2006; Choi et al., 2008; de Rougemont et al., 2011; Donaldson et al., 2008; Lindesmith et al., 2012; Prasad et al., 1999). Human noroviruses are highly resistant to harsh external environments. Repeated freeze−thaw process up to 14 cycles and long storage in frozen form up to 120 days did not have any notable effect on norovirus capsid integrity, as reflected by the measurement of the quantity of encapsulated virus RNA (Richards et al., 2012). In a volunteer challenge study, Norwalk virus inoculum treated with up to 10 mg/L of chlorine retained infectiousness as reflected by inducing typical symptoms of acute gastroenteritis (Keswick et al., 1985). Using in vitro-expressed virus-like particles, norovirus capsid was found to be highly stable in a wide range of pH from 3 to 7 and at elevated temperature up to nearly 60°C (Ausar et al., 2006). It was speculated that temperature, pH, and ionic strength affect the stability of the secondary and tertiary structure of the virus capsid protein, which in turn mediates virus infectivity and binding capability to inanimate surfaces (Samandoulgou et al., 2015).

4.3 GENOTYPES

4.3.1 OVERVIEW

Human noroviruses are still regarded as noncultivable despite a recent breakthrough that demonstrated limited human norovirus replication in B cells cocultured with bacteria expressing

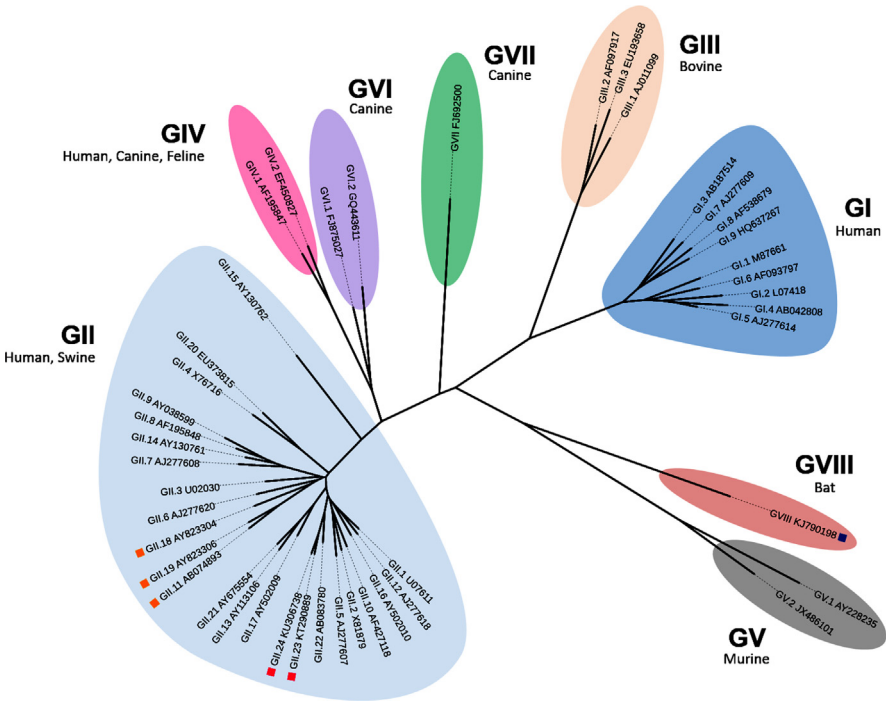

FIGURE 4.2

Classification of norovirus into eight genogroups (GI–GVIII) and 44 genotypes using neighbor-joining phylogenetic inference on complete amino acid sequences of VP1. One tentative new genogroup (GVIII from bats) and two tentative new genotypes (GII.23 and GII.24) not described in Vinje (2015) are indicated by blue (light gray in print versions) and magenta (dark gray in print versions) squares, respectively. Each sequence is denoted as genogroup.genotype or as only genogroup if there is just one genotype within the genogroup, followed by GenBank accession number. The animal host of each genogroup is shown in gray text underneath genogroup designation. All genotypes within GII infect humans except GII.11, GII.18, and GII.19, which infect pigs (black squares). For clarity, bootstrap values at nodes and branch lengths are omitted.

histo-blood group antigens (Jones et al., 2014, 2015). Because an efficient cell culture system is not available, serotyping of human noroviruses by neutralization assay is not yet possible; thus, "serotype" is not applicable to noroviruses. Currently, classification of norovirus is largely based on complete amino acid sequences of VP1 (Vinje, 2015; Zheng et al., 2006). Norovirus is genetically very diverse and can be classified into at least seven genogroups (GI–GVII) and 41 genotypes (e.g., GI.1) according to the latest scheme proposed by Dr. Jan Vinje of the National Calicivirus Laboratory of the US Centers for Disease Control and Prevention (CDC) (Vinje, 2015) (Fig. 4.2). Recently, an additional genogroup (GVIII) has been described (Wu et al., 2015). GI and GII infect humans only; GII.11, GII.18, and GII.19 infect porcine species. GIII infects bovine species. GIV infects canine and feline species. Human infections of GIV are very rare (Ao et al., 2014; Eden et al., 2012). GV infects murine species and is the only norovirus genogroup that can be cultivated in vitro efficiently in

macrophages and dendritic cells (Wobus et al., 2004). GVI and GVII infect canine species. Because genetic recombination at the ORF1/2 junction is common in norovirus (Eden et al., 2013; Giammanco et al., 2012; Lu et al., 2015b; Mans et al., 2014; Wong et al., 2013), a dual nomenclature system using both RdRp and VP1 sequences has been proposed in which RdRp genotype is denoted similarly as VP1 genotype but with a letter "P" preceding genotype number (e.g., GII.P3) (Kroneman et al., 2013). A norovirus strain with a GII.3 RdRp and a GII.6 VP1 will be designated as GII.P3_GII.6. Despite the broad genetic diversity, one peculiar genotype known as GII.4 has been predominant in human infections in both sporadic and outbreak settings during the past 20 years. The following section describes the molecular epidemiology of norovirus GII.4 and other important and emerging norovirus genotypes, as well as their relationship with foodborne outbreaks.

4.3.2 GII.4

The first strain that belonged to norovirus GII.4 was reported in outbreaks of gastroenteritis in a nursing home in Maryland in the winter of 1987–88 (Green et al., 2002). In a retrospective study on archived stool materials collected from hospitalized children between 1974 and 1991 in Washington, DC, GII.4 strains were found in samples as early as 1974 (Bok et al., 2009), suggesting that GII.4 strains have been circulating in humans for more than 40 years. Since then, sporadic detection has been reported, and the first pandemic GII.4 variant, known as 95/96-US, was reported in 1999 (Noel et al., 1999). Dating back to 1996, six pandemic GII.4 variants have been identified: 95/96-US (years of circulation, 1995–2002), Farmington Hills (2002–04), Hunter (2004–06), Den Haag (2006–09), New Orleans (2009 to present), and Sydney (2012 to present) (Pringle et al., 2015) (Fig. 4.3). A new GII.4 variant has emerged every 2–4 years, replacing the previously circulating GII.4 variant (Bull and White, 2011; Eden et al., 2013). Interestingly, the emergence of some new GII.4 variants has been associated with a surge in norovirus outbreaks in the community. For example, an international surveillance network of 10 European countries reported an unusual surge in norovirus gastroenteritis outbreaks in the summer of 2002 that temporally coincided with the emergence of the then novel GII.4 Farmington Hill variant (Lopman et al., 2004). Similar

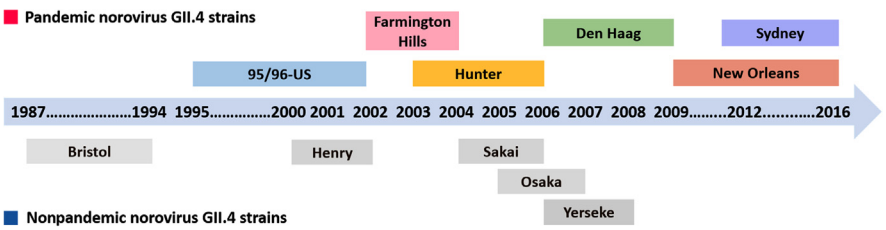

FIGURE 4.3

Schematic diagram showing the years of circulation of 11 key norovirus GII.4 variants detected since the 1980s. The long arrow in the middle denotes the timeline. Pandemic and nonpandemic GII.4 variants are shown above and below the timeline, respectively. Truncated years are indicated by serial dots. Some GII.4 variants have multiple names: Bristol (also known as Lordsdale, Camberwell, and MD145-12), 95/96-US (Grimsby), Farmington Hills (2002 variant), Hunter (2004 variant), Sakai (Chiba and Asia_2003), Yerseke (2006a and Laurens), and Den Haag (2006b and Minerva).

observations have been reported in other countries such as Australia (Bull et al., 2006). In 2006, atypical norovirus activities observed in Europe and Asia were associated with the emergence of the then novel GII.4 Den Haag variant (Ho et al., 2007; Kroneman et al., 2006). However, not all newly emerged GII.4 variants lead to increased norovirus activities in the community. In a study during the winter of 2009–10 in the United States, there was no increase in norovirus outbreaks amid of the emergence of the then novel GII.4 New Orleans variant (Yen et al., 2011). The current circulating GII.4 Sydney variant was also not found to associate with higher norovirus activity in the winter of 2012–13 in the United States (Leshem et al., 2013).

Norovirus GII.4 variants pose substantial disease burden of clinical significance in all age groups, especially young children and the elderly. In a 2-year cohort study of hospitalized norovirus gastroenteritis conducted between 2012 and 2014 in Hong Kong, 80% of cases were attributed to GII.4 variants (Chan et al., 2015b). A U-shaped age distribution in hospitalization incidence was observed, with the highest rate in young children aged 5 years or younger, followed by elderly aged older than 84 years. In a meta-analysis of more than 200 published articles on norovirus outbreaks of different virus genogroups and genotypes, GII.4 was found to be associated with more severe clinical manifestations in terms of hospitalization and death rates (Desai et al., 2012). Elderly aged older than 84 years are at risk of fatal norovirus infections (van Asten et al., 2012). Norovirus GII.4 also exhibits a higher concentration of fecal virus shedding compared to other genotypes (Costantini et al., 2016). The clinical relevance of fecal viral concentration remains largely elusive and contradictory. In an observational study of 40 inpatients hospitalized with norovirus GII.4 infections, longer duration of diarrhea was observed in cases with higher fecal viral concentration (Lee et al., 2007). However, in a retrospective study of nosocomial outbreaks, no correlation between symptom duration and fecal viral concentration was observed (Partridge et al., 2012).

4.3.3 GII.17

Norovirus GII.17 has been the focus of the field recently. The story started from a report that found that GII.17 accounted for more than 80% of outbreaks of gastroenteritis in different settings (kindergartens, colleges, and factories) among 10 cities in Guangdong province of China in the winter of 2014–15 (Lu et al., 2015a). This is in sharp contrast to the previous winter of 2013–14, during which GII.4 Sydney 2012 was predominant. Phylogenetic analysis suggested that the emergent GII.17 was a new variant, and it was named Kawasaki 2014 (also known as Kawasaki308-like) by the NoroNet (de Graaf et al., 2015). A similar surge in activity of the emerging norovirus GII.17 in the same period was reported in other cities in China, including Beijing, Hong Kong, Huzhou, Jiangsu, and Shanghai (Chan et al., 2015a; Chen et al., 2015; Fu et al., 2015; Gao et al., 2015; Han et al., 2015). This new variant was also associated with a waterborne outbreak in Hebei, China (Qin et al., 2016). Displacement of the contemporary GII.4 Sydney 2012 by the GII.17 Kawasaki 2014 outside China, however, was reported only in Japan (Matsushima et al., 2015). Although there were reports of sporadic detection of GII.17 Kawasaki 2014 in North America and Europe, large-scale outbreaks or evidence of GII.4 displacement have been lacking (Dinu et al., 2016; Medici et al., 2015; Parra and Green, 2015). The current geographical restriction of GII.17 Kawasaki 2014 to a portion of Asia is intriguing. It will be very interesting to determine whether or not this new GII.17 variant will persist in the 2016–17 winter season in China and whether it will spread globally in a way resembling the pandemic GII.4 strains.

Our current understanding of this previously rare norovirus GII.17 genotype is very limited. Prior to the sudden emergence and predominance in China, norovirus GII.17 genotype had been only sporadically reported in clinical cases (de Graaf et al., 2015). One study reported frequent detection of norovirus GII.17 in environmental water samples (Kiulia et al., 2014). Currently, several pieces of evidence suggest that the emergent GII.17 Kawasaki 2014 is of public health concern. First, older children and adults aged 5−65 years comprise a high proportion (up to 50%) of observed hospitalized cases of gastroenteritis infected with the new GII.17 variant (Chan et al., 2015a; Chen et al., 2015). This shift in age distribution suggests that GII.17 may be associated with a higher susceptibility or more severe clinical presentation in older children and adults. GII.17 Kawasaki 2014 may be able to escape immunity acquired from previous GII.4 infections. This speculation is supported by recent elucidation of the crystal structure of this new GII.17 variant (Singh et al., 2016). Frequent hospitalization of immunocompetent older children and adults may also reflect higher virulence of this new GII.17 variant. Second, saliva binding analysis reveals that the new GII.17 Kawasaki 2014 is capable of recognizing a wide spectrum of histo-blood group antigens present on the host cell surface that can serve as an attachment factor for noroviruses, suggesting an expanded susceptible human population for GII.17 (Chan et al., 2015a; Zhang et al., 2015). Third, using molecular clock analysis on complete VP1 sequences, norovirus GII.17 was found to evolve at a rate one order of magnitude faster than that of GII.4 during the past decade (Chan et al., 2015a). Although the mechanism remains elusive, the fast-mutating nature of GII.17 will fuel norovirus GII.17 with a high potential to further acquire virulence and transmissibility in the future. International collaborative effort to monitor the global spread of this emerging GII.17 variant is urgently needed.

4.3.4 NEW GENOGROUPS AND GENOTYPES

The first systematic classification of noroviruses into five genogroups (GI−GV) was reported in 2006 (Zheng et al., 2006). Since then, three tentatively new genogroups, GVI−GVIII, have been identified (Fig. 4.1). Using the traditional Sanger sequencing approach, two canine norovirus-like sequences were identified from fecal matter of diarrheal dogs and were tentatively designated GVI and GVII (Martella et al., 2008; Tse et al., 2012). Recently, with advancements in the increasingly affordable high-throughput next-generation sequencing (NGS) technology, human and animal viromes are being rapidly explored and sequenced (Berg et al., 2015; Hoffmann et al., 2015; Sasaki et al., 2014; Woo et al., 2014). In a study of bat virome using anal and pharyngeal swab samples collected from more than 4000 bats representing 40 bat species in China, six bat calicivirus-like sequences were identified, including two that were most closely related to noroviruses (Wu et al., 2015). Phylogenetic analysis suggests that the bat sequences may represent a novel norovirus genogroup, tentatively designated GVIII. It may seem that the search for novel noroviruses has been more fruitful in nonhuman animal species. This is not true. In a community-based cohort of Ecuadorian children, a norovirus belonging to a novel GII genotype, tentatively called GII.23, was reported (Lopman et al., 2015). In 2016, a complete norovirus genome (GenBank accession number KU306738), belonging to another novel GII genotype, tentatively called GII.24, was released in GenBank by the National Calicivirus Laboratory of the CDC. Currently, there is very little information about this GII.24 genome except that it was collected from a stool sample in Nicaragua and was sequenced using NGS. It is anticipated that the wide utilization of state-of-the-art molecular

sequencing technology in both research and clinical laboratories will reveal more norovirus strains and expand our understanding of the species distribution of noroviruses in the future.

4.3.5 NOROVIRUS GENOTYPE AND FOODBORNE OUTBREAKS

One characteristic feature of norovirus-associated foodborne outbreaks is the presence of multiple norovirus genotypes. In a meta-analysis of data submitted to FBVE/NoroNet of the Netherlands, CaliciNet of the CDC, and ESR-Epi-Surv of New Zealand from 1999 to 2012, 10% of foodborne outbreaks were attributed to genotype GII.4 and 27% to other single non-GII.4 genotypes, whereas 37% were caused by mixed GII.4 and non-GII.4 genotypes (Verhoef et al., 2015). It also appears that non-GII.4 genotypes are more likely to associate with foodborne outbreaks. In an analysis of 3960 norovirus outbreaks reported to CaliciNet of the CDC between 2009 and 2013, several non-GII.4 genotypes in genogroup I (GI.3, GI.6, and GI.7) and genogroup II (GII.3, GII.6, and GII.12) were found to associate with foodborne outbreaks (Vega et al., 2014). Oyster- and other shellfish-associated foodborne outbreaks are notoriously known to involve multiple norovirus genotypes, including GI.1, GI.2, GI.4, GI.5, GI.6, GI.7, GII.3, GII.4, GII.6, GII.7, GII.11, GII.12, GII.13, GII.14, and GII.17 (Cho et al., 2016; Ma et al., 2013; Rajko-Nenow et al., 2013, 2014; Wang et al., 2015). This is because norovirus can bind to histo-blood group antigens present on the intestinal cells of oysters and other bivalves, including clams and mussels (Tian et al., 2006, 2007, 2008). The filter-feeding nature of oysters also leads to bioaccumulation of noroviruses from the living water environment contaminated with human sewage. In contrast, foodborne outbreaks associated with other food types such as fruits are more likely to involve a single norovirus non-GII.4 genotype (Hoffmann et al., 2013; Muller et al., 2015; Ruan et al., 2013), probably via contamination from asymptomatic, norovirus-shedding food handlers (Barrabeig et al., 2010; Franck et al., 2015).

4.4 CONCLUSIONS AND PERSPECTIVES

Human noroviruses are ubiquitous and highly infectious. Structural determinants of their super environmental stability remain poorly understood, partly due to the lack of a robust and efficient in vitro culture system to assess virus infectivity. Research priority should be given to optimize existing norovirus culture models. The mechanism of norovirus adsorption onto food surfaces is a largely unexplored research area. Given that some norovirus genotypes are more likely to be found in foodborne outbreaks, one may assume that different norovirus genotypes have different preferences for attachment factors on food surfaces. What are these factors? Are they proteins or carbohydrates or both? Noroviruses are found in a diverse array of mammalian species, including bats, which are long-recognized super-carriers of many ancient viruses such as coronaviruses. This suggests that noroviruses may have an ancient origin. Some animal noroviruses (e.g., porcine GII.11, GII.18, and GII.19) are closely related to human noroviruses. Although there are no reports of human infections of animal noroviruses, zoonotic potential of norovirus transmission from other mammals, especially those farmed for human consumption, to humans via the food chain cannot be neglected. The prevalence of noroviruses in animal species needs to be systematically monitored.

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