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Human Norovirus as a Foodborne Pathogen: Challenges and Developments

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Abstract

Human noroviruses (NoVs) are the leading cause of foodborne illness in the United States, and they exact a considerable human and economic burden worldwide. In fact, the many challenging aspects of human NoV have caused some to call it the nearly perfect foodborne pathogen. In this review, a brief overview of NoVs and their genetic structure is provided. Additionally, the challenges and recent developments related to human NoVs regarding viral evolution, transmission, epidemiology, outbreak identification, cultivation, animal and human models, and detection are presented.

1. INTRODUCTION

Human noroviruses (NoVs) are the most common cause of acute gastroenteritis and are responsible for substantial morbidity and mortality worldwide (Patel et al. 2008, Glass et al. 2009). One important mode of NoV infection is via contaminated food, as these viruses are considered the leading known foodborne agent in the United States, accounting for over 50% of foodborne illnesses annually (Scallan et al. 2011b). However, foodborne disease of unknown etiology is estimated to account for as much as 80% of all food-related illness, of which human NoVs are likely a considerable portion (Scallan et al. 2011a).

NoVs are a member of the family *Caliciviridae*, which derives its name from the Greek word for cup (*calyx*), in reference to cup-like depressions on the surface of the virus (Glass et al. 2009). The *Caliciviridae* family contains six genera: *Norovirus*, *Vesivirus*, *Lagovirus*, *Recovirus*, *Sapovirus*, and *Becovirus*. The *Norovirus* genus is the most common cause of disease in humans relative to the other five. These viruses were previously referred to by other names, such as "small round structured viruses" and "Norwalk-like viruses" (Lambden et al. 1993). They are typically 27–37 nm in diameter, nonenveloped, and have a 7.5–7.7-kilobase positive-sense single-stranded RNA genome that consists of three open reading frames (ORFs) (Glass et al. 2000, Jiang et al. 1993, Lambden et al. 1993). ORF1 codes for a nonstructural polyprotein that is believed to self-cleave into six to seven proteins (NS1–NS7). Some of the nonstructural proteins include an NTPase (Pfister & Wimmer 2001), a protease (Blakeney et al. 2003, Liu et al. 1996), an RNA-dependent RNA polymerase (Fukushi et al. 2004, Rohayem et al. 2006), and a protein (VPg) that is covalently linked to the 5′ end of the genome and is suspected to initiate translation and possibly transcription (Subba-Reddy et al. 2011, Belliot et al. 2008, Daughenbaugh et al. 2003, Goodfellow et al. 2005). For a more thorough discussion of NoV genomic structure and function, refer to Thorne & Goodfellow (2014).

ORF2 and ORF3 encode major (VP1) and minor (VP2) capsid proteins, respectively (Glass et al. 2000, Prasad et al. 1999). The VP1 protein contains internal N-terminal and shell (S) domains as well as a protruding (P) domain comprised of a P2 subdomain that is the most exposed part of the virus; the P1 subdomain lies below P2 (closer to the S domain). The viral capsid is a $T = 3$ icosahedron that contains 180 copies of VP1, which form dimers. When expressed in certain models, the proteins self-assemble in empty capsids called virus-like particles (VLPs; Jiang et al. 1992). Human NoVs cannot be cultivated in vitro, and there is no known animal model for reliable virus propagation outside of human beings.

Historically, NoVs have been classified based on the degree of difference between the amino acid sequences of the VP1 major capsid protein (Zheng et al. 2006). One advantage of using this protein is that it contains the highly variable P2 subdomain that is suspected to interact with specific host cell targets such as the histo-blood group antigens (HBGAs) and possibly others (Huang et al. 2003, Murakami et al. 2013). For a more comprehensive review of putative NoV host cell receptors, refer to Huang et al. (2005) and Donaldson et al. (2010). Historically, researchers have defined genogroups as having 45–61% uncorrected pairwise difference in the VP1 region (Zheng et al. 2006). Accordingly, the *Norovirus* genus has been divided into six genogroups (GI–GVI), three of which cause disease in humans (GI, GII, and GIV). The recent GVI and a new tentatively proposed GVII genogroup consist of canine NoV (Martella et al. 2008, Tse et al. 2012). Further subdivisions within genogroups, representing genetic clusters, constitute genotypes. Researchers have defined genotypes as containing 14–44% VP1 amino acid difference, whereas strains have 0–14% difference (Zheng et al. 2006). To date, over 31 genotypes of human NoV have been reported, of which 9 and 22 belong to GI and GII, respectively. At the time of this writing, this constitutes over 150 strains, but genotypic and strain designations are evolving.

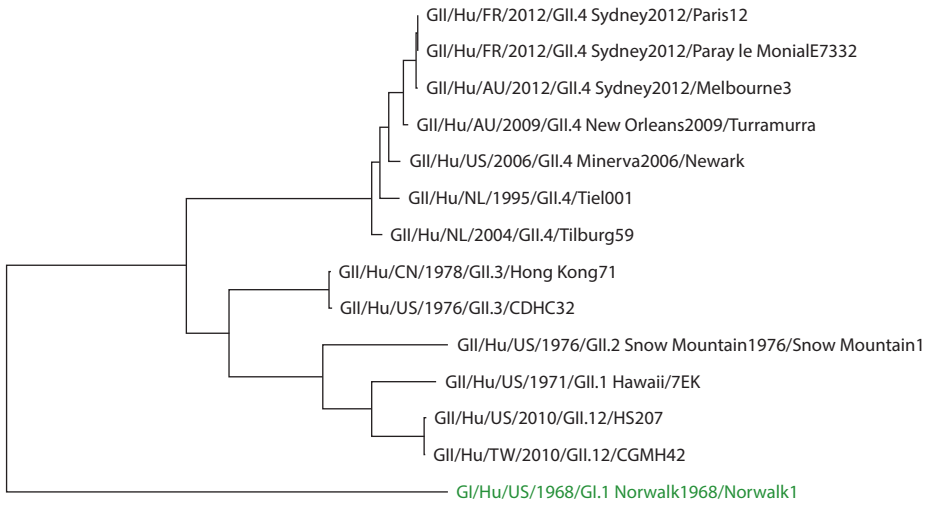

Figure 1

Phylogenetic tree of selected GII strains. The tree was constructed based on only full-length VP1 sequences and contains the prototype GI.1 Norwalk virus (*green*) as an example of GI. Interested readers are referred to Kroneman et al. (2013) and Vinjé (2015) for more comprehensive coverage of norovirus phylogeny and genotyping. Abbreviations: GI, genogroup I; GII, genogroup II; VP1, viral protein 1 (the major capsid protein).

Traditional nomenclature has been based on genogroup-genotype combination (e.g., GII/4 or GII.4). With the discovery that recombination can occur in the ORF1-ORF2 junction and other areas, and with identification of more genotypes and strains, researchers have proposed an alternative international standard for strain classification. This assignment is a dual typing system based on specific difference cutoffs of the partial RdRp (ORF1, 1,300 nucleotides) and complete VP1 (ORF2) sequences. The new nomenclature of an identified isolate would be written as norovirus Genogroup/Host/Country/Isolation Year/Partial ORF1_ORF2_Strain Name and Index Year/Isolate City Name (possibly with a unique isolate number). For example, an isolate of genogroup II found in Houston, Texas (United States), that has a GII.P4 partial ORF1 grouping with a GII.4 Hunter ORF2 sequence would be designated as norovirus GII/Hu/US/2005/GII.P4_GII.4_Hunter2004/Houston23. If only ORF2 is known, then it would be norovirus GII/Hu/US/2005/GII.4_Hunter2004/Houston23 (Kroneman et al. 2013). An example phylogenetic tree of some GII human NoV strains is provided in **Figure 1**.

Several features of NoVs make them highly transmissible. First, the infectious dose is low; estimates range from 20 to several thousand particles, depending on the study and corresponding modeling assumptions (Teunis et al. 2008, Atmar et al. 2013). Also, these viruses are antigenically and genetically diverse, resulting in many different strains with limited immunological cross-protection between strains. Although still debatable, evidence suggests that immunity to any one strain may be as short as a few months to two years (Parrino et al. 1977). Human NoVs persist in the environment, lasting weeks to years, depending on environmental conditions such as temperature and relative humidity. They are also resistant to nearly all of the active ingredients in cleaning products, sanitizers, and disinfectants commonly used in food production and processing, including quaternary ammonium compounds, detergents, alcohols, and even chlorine at regulated concentrations. The same can be said about food processing and preservation methods such as

heat, ionizing radiation, organic acids, preservatives, and manipulation of pH or water activity. These issues are discussed in multiple reviews (Hirneisen et al. 2010, Hoelzer et al. 2013, Li et al. 2012b). Some call human NoVs the near-perfect foodborne pathogen, except for the fact that it cannot multiply (but does persist) in foods and the environment.

Many unknowns remain for this important foodborne pathogen, and food virology is an area of active research. In this review, we discuss efforts to address several recalcitrant issues associated with the study and understanding of NoVs, with a particular focus on the foodborne transmission route. These topics include efforts in human NoV cultivation, recent epidemiological findings, strain evolution, and key detection conundrums. The reader will come away with a better understanding of why this foodborne pathogen is so difficult to study and control and how scientists are working to address these issues.

2. IN VITRO AND IN VIVO CULTIVATION

2.1. In Vitro Cultivation of Human Norovirus

The single most important limitation on the ability to study human NoV is the lack of an in vitro cultivation method, despite almost 50 years of attempts. Cultivation using many mammalian cell lines and human NoV strains has been attempted. For example, Duizer et al. (2004) tested A549, AGS, Caco-2, CCD-18, CRFK, CR-PEC, Detroit 551, Detroit 562, FRhK-4, HCT-8, HEC, HeLa, HEp-2, Ht-29, HuTu-80, I-407, IEC-6, IEC-18, Kato-3, L20B, MA104, MDBK, MDCK, RD, TMK, Vero, and 293 cell lines, along with about 33 different human NoV strains, all leading to no significant findings. Contrary to the case for murine NoV (GV), human macrophages and dendritic cells were unable to support infection by human NoV strains (Lay et al. 2010). Guix et al. (2007) transfected Norwalk virus (the representative GI.1 strain) RNA in the tumorigenic human liver Huh-7 cells, achieving viral replication through one cycle but nothing thereafter, suggesting cultivation issues occur in the attachment and uncoating stages of replication. Overexpression of the gene responsible for producing an HBGA (the H antigen) in Huh-7 cells resulted in enhanced binding of Norwalk virus but failed to produce viral infection. Furthermore, mutant Huh-7.5.1 cells with an inactive RIG-I (a host protein that combats alien RNA in cells) supported viral replication through only one cycle as well. Based on these findings, in vitro cultivation issues appear to be at the attachment and uncoating stages of infection. This study implicated the viral VPg as necessary for viral replication (Guix et al. 2007). Some initial success was observed when challenging adult duodenal tissues in ex vivo culture. However, when using the in vitro human glandular epithelial cell line HIEC-6, only low (2 $\log_{10}$ or less) viral RNA production was observed, as was the absence of a cytopathic effect (Leung et al. 2010).

A three-dimensional (3-D) cell culture model was reported almost a decade ago for human NoV cultivation. Human small intestinal cells were grown on collagen I–coated porous microcarrier beads in a rotating wall vessel incubator (Straub et al. 2007). This mimics the fluid-shear environment of the intestine and thus allows the intestinal epithelium to grow and differentiate in three dimensions. Straub et al. (2007) showed replication of two GII and one GI human NoV strains in five passages using this model. The same group used the 3-D cell culture method in Caco-2 cells and seemed to have some limited success (Straub et al. 2011). However, coordinated international efforts have failed to replicate these findings (Herbst-Kralovetz et al. 2013, Papafragkou et al. 2013, Takanashi et al. 2014). More recent efforts focus on the use of human intestinal enteroids or organoids, derived from human intestinal epithelial stem cells that are created ex vivo into 3-D small intestinal epithelial functional units. These are composed of the entire villus-crypt axis and epithelial cell types normally present in the small intestine (Kovbasnjuk et al. 2013). A report by

Jones et al. (2014), recent at the time of this writing, describes the successful replication of a GII.4 human NoV strain in human B cells. The data suggest that enteric bacteria containing HBGA-like molecules are cofactors for infection, but additional work is needed to validate these findings. For additional information on in vitro human NoV propagation, several review articles are available (Karst et al. 2014, Li et al. 2012b, Richards 2012, Vashist et al. 2009).

2.2. Animal Models for Human Norovirus

Several animal models have been proposed for human NoV propagation. One of these is gnotobiotic pigs, which, when challenged with a GII.4 strain, appear to exhibit mild diarrhea, shed virus, support some degree of viral replication, and exhibit a specific immune response profile (Cheetham et al. 2006). Additionally, the binding patterns of different human NoV VLPs to HBGA-expressing gnotobiotic pig buccal and duodenal tissues have been observed (Cheetham et al. 2007). Gnotobiotic calves have also been reported to host human NoV GII.4 infection (Souza et al. 2008).

The presence of GI and GII human NoV antibodies has been reported in several nonhuman primate species (Jiang et al. 2004). Rockx et al. (2005) found some evidence that rhesus macaques had the potential to serve as a model when inoculated with Norwalk virus (GI.1). Chimpanzees were also shown to host infection, harbor viral antigen production, and shed virus when injected intravenously with filtered Norwalk virus (Bok et al. 2011).

A recent study reported that some strains of BALB/c mice that had been "humanized" (grafted with certain human stem cells) and BALB/c mice that had not been humanized were able to host replication of a human NoV GII.4 strain (Taube et al. 2013). This report is significant because it presents the possibility of a smaller, less cumbersome animal model for human NoV research. However, in this case, viral introduction was interperotineal, not oral; the immunocompromised state of the mice eliminates applicability to immunological studies; and the animals shedding virus were asymptomatic. Further support for, replication of, and improvement of this model are needed. Suffice it to say that no animal model yet produces a response to challenge with human NoV that is similar to that of humans.

2.3. Human Challenge Studies

In the absence of cell culture or animal models, scientists have resorted to human challenge studies. Perhaps the first example of a human NoV challenge study occurred shortly after the original 1968 Norwalk virus outbreak; in this study, fecal specimens derived from secondary cases were diluted and fed to volunteers (Dolin et al. 1972). Some important fundamental questions about human NoV infection have been addressed using human feeding studies. For instance, the aforementioned information on NoV infectious dose was derived from human challenge data (Atmar et al. 2013, Teunis et al. 2008). Other information was also elucidated from these studies, as Atmar et al. (2013) found that the incubation period of the virus ranged from 20 to 50 h, with some association of a shorter incubation period for higher inoculation doses. Furthermore, symptom duration ranged from 8 to 60 h; however, viral shedding of infected patients ranged from 6 to 55 days. Viral shedding in vomitus was also reported, with a median of 41,000 genomic equivalents of NoV per mL of vomitus. This is compared to medians of 160 billion and 10 billion genomic equivalents per mL in the stool of people who exhibited gastroenteritis symptoms and those who did not, respectively (Atmar et al. 2013).

Human feeding has also been used as a way to measure disinfection efficacy in high-pressure processing of oysters (Leon et al. 2011) and virus carriage on the hands of infected individuals

(Liu et al. 2013), both important issues in the realm of food safety. In a recent study, serum samples from volunteers previously challenged with the Snow Mountain virus (GII.2) were used to characterize human NoV strain evolution and antigenic and immune response characteristics (Lindesmith et al. 2005, Swanstrom et al. 2014). Although the data obtained by human challenge studies are incredibly valuable, these studies are expensive, time consuming, and require rigorous regulatory approvals. They must therefore be carefully designed to optimize data collection but are, nonetheless, only rarely done.

2.4. Cultivable Human Norovirus Surrogates

In the absence of a cultivable human NoV strain, cultivable surrogate viruses have been widely used. These include feline calicivirus (FCV; Doultree et al. 1999), murine NoV (MNV; Wobus et al. 2006), Tulane virus (TV; Farkas et al. 2008), rabbit hemorrhagic disease virus (Meyers et al. 1991), porcine enteric calicivirus (Flynn & Saif 1988), poliovirus, hepatitis A virus, and MS2 bacteriophage (Bae & Schwab 2008, Shin & Sobsey 2003). To date, FCV propagated in Crandell-Reese feline kidney cells and MNV propagated in mouse macrophage RAW 264.7 cells are the most widely used surrogates. FCV is a member of the *Vesivirus* genus in the *Caliciviridae* family and causes a respiratory disease in cats but is considered appropriate because it is a member of the same family as *Norovirus* and is of similar genomic structure, at least by the standards of the late 1990s, when it was first used. FCV is still considered the gold standard by the US Environmental Protection Agency for products seeking to make antinoroviral claims. MNV was reported by Karst et al. (2003) about a decade ago and was rapidly adopted because it is a member of the *Norovirus* genus (categorized as a GV NoV) and hence has more similar genetic and structural characteristics to human NoV than other surrogates. However, it causes gastroenteritis and meningitis in immunodeficient mice only (Kahan et al. 2011).

Many studies have evaluated the environmental behavior of human NoVs based on that of the cultivable surrogate viruses. Almost all these studies have used one or a few surrogates and failed to make direct comparisons between the behavior of the surrogates to human NoVs, in part because the latter cannot be cultivated in vitro. A full accounting of these studies is available elsewhere (Baert et al. 2009a, Hirneisen et al. 2010, Hoelzer et al. 2013). Differences in susceptibility of the surrogates to environmental conditions and inactivation treatments are now well documented. Richards (2012) provides a critical review of these issues. Many of the surrogates differ in terms of resistance to elevated temperature, extremes of pH, and susceptibility to organic solvents as well as various chemical sanitizers and disinfectants. Generally, FCV appears to be more susceptible to pH and chlorine than MNV, whereas MNV is more susceptible to alcohols (Cannon et al. 2006, Park et al. 2010, Tung et al. 2013). A recent systematic review and meta-analysis of studies using two or more surrogates concluded that MNV, hepatitis A virus, and bacteriophage MS2 were generally more stable than FCV to chemical disinfectants, but by an average of ≤ 1.5 $\log_{10}$ plaque-forming units (Hoelzer et al. 2013).

TV is a newer surrogate, first reported by Farkas et al. (2008). TV was isolated from the stool of captive rhesus macaques and was phylogenetically classified into a new *Caliciviridae* genus called *Recovirus*. The TV genome has a general genetic similarity to *Norovirus*, and the capsid has a similar size (~36 nm) and buoyant density as other members of the *Caliciviridae* family (Farkas et al. 2008). Unlike MNV and FCV, TV has been reported to bind HBGA types A and B (Farkas et al. 2010). This is significant in that these HBGAs are the same putative host receptor/cofactor as human NoVs, suggesting that TV may be a more relevant surrogate in cases in which capsid functionality and behavior (disinfection studies) are being assessed. TV is readily cultured in the LLC-MK2 monkey kidney cell line.

Recent studies have sought to characterize the susceptibility of TV to different inactivation strategies. One such study compared TV and MNV sensitivity to pH, chlorine, heat, and survival in tap water at room and refrigeration temperatures. MNV was found to be more stable in refrigerated tap water and at various pH values compared to TV. However, MNV and TV behaved similarly in 20°C tap water, under different heat treatments (50–75°C for 2 min), and at most concentrations of chlorine (0.2, 20, 200, and 2,000 ppm) (Hirneisen & Kniel 2013a). In another study, MNV and TV showed inactivation profiles similar to human NoV [assessed by reverse transcriptase quantitative polymerase chain reaction (RT-qPCR)] when inoculated on spinach for up to a week (Hirneisen & Kniel 2013b). Similarly, Wang et al. (2013) observed no statistically significant differences in the survival of MNV, TV, and hepatitis A virus on alfalfa seeds and sprouts held at 22°C for 20 days. Certainly, further work must be done to identify the appropriate, most conservative (e.g., resistant) surrogate to be used for a given condition or treatment. The choice of an appropriate surrogate will likely be a function of the experimental question being posed. Comparative studies in which the behavior of surrogates is compared to that of human NoV (using RT-qPCR) are also needed. The lack of a universally applicable cultivable surrogate for which there is a clear relationship to the behavior of human NoVs under a variety of circumstances has hindered progress in understanding the efficacy of inactivation strategies.

3. UNDERSTANDING THE EPIDEMIOLOGY OF NOROVIRUS DISEASE

3.1. Recent Estimates of Human Norovirus Disease Burden

NoVs are the leading cause of acute viral gastroenteritis and of foodborne disease in most—if not all—of the world. But food is only one means by which the virus is spread, and person-to-person transmission remains the most common route. Recently, person-to-person transmission was reported to account for 62–84% of all reported outbreaks (Vega et al. 2011, 2014). The overall burden of disease from all transmission routes combined is staggering. A compilation of different studies of incidence and outcome rates produced estimates that NoVs are responsible for 19–21 million illnesses, 1.7–1.9 million outpatient visits, 400,000 emergency department visits, 56,000–71,000 hospitalizations, and 570–800 deaths annually in the United States alone. When disease rates are applied to a US resident who lives to the age of 79 years, he or she would experience five incidents of human NoV disease in a lifetime, have a 1 in 2 chance of a disease-related outpatient visit, a 1 in 50–70 chance of hospitalization, and a 1 in 5,000–7,000 chance of death due to NoV infection (Hall et al. 2013, Scallan et al. 2011b). Long-term care facilities are particularly important in transmission, constituting 62.5% (2,475/3,960) of NoV cases reported to the United States Centers for Disease Control and Prevention (CDC) from 2009 to 2013 (although most NoV cases are not reported to the CDC) (Vega et al. 2014). European statistics are generally similar to those from the United States (Phillips et al. 2010, Baert et al. 2009b, Tam et al. 2012).

In outbreak settings, GII infections outnumber GI infections, as GII strains have been found to occur in 80% or more of outbreaks. The epidemic strains belonging to GII.4 are responsible for the vast majority of cases, particularly those associated with person-to-person transmission (Matthews et al. 2012, Vega et al. 2014). For example, 72% of long-term care facility outbreaks reported from 2009 to 2013 were caused by GII.4 strains, but non-GII.4 strains also caused considerable morbidity, especially in association with restaurant and food-related settings (Vega et al. 2014). Human NoV disease incidence appears to have increased of late in multiple countries, probably because of the emergence of a new GII.4 strain (the Sydney strain, which appeared first in 2012) (van Beek et al. 2013). In fact, human NoV mortality can spike by as much as 50% during seasons in which an emergent epidemic (GII.4) strain is circulating (Hall et al. 2012a).

3.2. The Burden of Foodborne Norovirus Disease

Limited statistics are available relative to the amount of NoV disease attributable to foods. Obtaining these estimates is extremely challenging because multiple factors must be considered and assumptions made to generate population-level estimates of the burden of foodborne illness. For laboratory-confirmed surveillance, for example, the infected patient must seek medical attention, a clinical sample must be obtained, a laboratory must identify the pathogen, and the illness must be correctly reported up the chain of health authorities (Scallan et al. 2011b). Unfortunately, disease associated with human NoVs is widespread, frequently goes undiagnosed, is not normally life threatening, and is not commonly reported to public health authorities. Consequently, foodborne disease incidence estimates for NoV are largely determined based on population-level data and mathematical modeling. The most commonly cited US statistic is that of Scallan et al. (2011b), who estimated that viruses account for 59% of these foodborne diseases and that NoV account for 99% (5.5 million) of all viral foodborne illness incidents per year. Although the illness is usually self-limiting with infrequent death, the sheer numbers result in about 15,000 hospitalizations (26%, second in foodborne hospitalizations) and 150 deaths (11%, fourth in foodborne deaths) annually (Scallan et al. 2011b). In Canada, NoV is also considered the leading cause of foodborne illness, accounting for 65% of known illnesses (Thomas et al. 2013).

However, estimates of the comparative importance of food relative to other transmission routes vary widely. In a study of outbreak data published from 1983 to 2011, Matthews et al. (2012) suggested that the majority of NoV infections were transmitted by foodborne routes (54%), with person-to-person ranking second (26%). However, this was a meta-analysis of published outbreaks and not necessarily based on population-based surveillance data. Using US CaliciNet surveillance system data from 2009 to 2013, Vega et al. (2014) concluded that 83.7% of NoV outbreaks were person-to-person, whereas only 16.1% were foodborne. In another study focused only on foodborne outbreaks, 46% (3,000) of approximately 6,300 outbreaks of known cause reported to the CDC between 2001 and 2008 were attributed to NoV (Hall et al. 2012b).

3.3. Identifying and Tracing Norovirus Outbreaks

Human NoV outbreaks are difficult to identify and even more difficult to trace back to a common food source. In addition to the general problems described above, most foodborne NoV outbreaks are small and associated with a retail setting (and hence investigated locally or regionally), and secondary person-to-person spread (propagated outbreaks) is very common. Even when a foodborne source is identified, many factors hinder detection of the virus in the food (discussed below).

Investigators have recently applied some novel approaches to human NoV outbreak identification. Verhoef et al. (2011) used sequence analysis of NoV genetic clusters, in conjunction with epidemiological data, to retrospectively identify common source outbreaks occurring in Europe from 1999 to 2008. They found a notable increase in common source and international outbreaks compared to the numbers identified originally by the Foodborne Viruses in Europe network (Verhoef et al. 2011). In a later study, the same team analyzed 500 publicly available, full-length NoV capsid sequences for outbreak identification. Not surprisingly, a region containing most of the P2 subdomain (nucleotide positions 900–1,400) was found to be the best for assigning genotypes, recognizing variants, and identifying outbreak events. Within this region, a minimum fragment of 950 to 1,350 nucleotides of the capsid gene could be used to identify more than 80% of outbreak events (Verhoef et al. 2012). A similar approach has been taken in tracing and identifying

nosocomial infections (Sukhrie et al. 2011). With a distinctly foodborne focus, Verhoef et al. (2010) used genotype frequency distributions from NoVs isolated from European bivalve mollusks, in conjunction with genotype frequency derived from human illness surveillance, to differentiate outbreaks caused by contamination early in the food chain (i.e., during production or processing) versus those occurring later (i.e., food-handler-associated). In a completely different approach, Rha et al. (2013) used BioSense, a national, electronic, US health-care surveillance system, to statistically correlate emergency department chief complaint data to seasonal increases of human NoV disease, proposing this method as a means of detecting the beginning of the NoV outbreak season (Rha et al. 2013). Clearly, the combination of classic epidemiological outbreak investigation with nucleic acid sequence analysis, health-care surveillance, or both increases the resolution of investigations.

3.4. Food Attribution and Transmission Estimates for Norovirus

Another epidemiological challenge related to NoVs—as well as other foodborne pathogens—is attribution of cases or outbreaks to specific food commodities. Because surveillance data are so sparse, many attribution studies are based largely on outbreak data. Over the past several years, the CDC has embarked on more comprehensive human NoV food attribution studies. Using data from 1998 to 2008 obtained from the Foodborne Disease Outbreak Surveillance System, they have made some general conclusions. First, the majority of food-associated NoV outbreaks in their database could not be attributed to one or more specific foods. Foods to which outbreaks could be attributed could be subdivided into multicomponent or complex foods and single component or simple foods (Painter et al. 2013). Human NoV outbreaks were more often caused by complex foods relative to simple foods. Further breakdown of the simple foods suggested that fresh vegetables were responsible for about 30–40% of simple food outbreaks, followed by fruits and nuts (10–20%), mollusks (10–15%), and dairy (5–15%) (Gould et al. 2013; Hall et al. 2012b, 2014; Painter et al. 2013). Very similar results were obtained using 2009–2012 data (Hall et al. 2014) and by others not affiliated with the CDC (Batz et al. 2012, Greig & Ravel 2009).

Of the NoV outbreaks that could be attributed to a single location, restaurants and delicatessens were the most common (63–64%), followed by catering and banquet halls (11–17%) and private homes (4–6%) (Hall et al. 2012b, 2014). Postharvest contamination by food handlers during preparation of foods was suspected as a possible source in as much as 90% of foodborne NoV outbreaks (Hall et al. 2014) and was specifically implicated as the source of contamination in 53% of outbreaks (Hall et al. 2012b). However, in many instances, the source (pre- or postharvest) could not be resolved. Not unexpectedly, the only food for which preharvest contamination constituted a notable proportion of overall contamination was mollusks (Hall et al. 2012b). Of the 528 foodborne NoV outbreaks reported during 2009–2012 for which specific contamination factors were identified, infectious workers were responsible for 70% of these outbreaks, and bare-hand contact with ready-to-eat foods was identified in 54% of them (Hall et al. 2014). Clearly, the food handler appears to be responsible for the majority of foodborne outbreaks.

In conclusion, the majority of NoV outbreaks appear to be transmitted between individuals, although foodborne transmission is also significant. Estimates of the relative proportion of food-associated transmission vary widely, and more refined data are needed. Complex, prepared, ready-to-eat foods are overrepresented in outbreaks, and the most common setting for these is restaurants, delicatessens, and catering facilities, implicating food handling as the likely contamination source. In the case of simple foods, vegetables, fruits, and nuts are the most likely culprits, but it is difficult to discern how important preharvest contamination is in these products. Even

in the absence of comprehensive data, targeting the food preparation sector appears to be an efficacious way to address the foodborne NoV problem.

4. NOROVIRUS EVOLUTION AND EPIDEMIC STRAIN EMERGENCE

In the past two decades, the GII.4 genotype has emerged as the cause of the majority of human NoV cases (Lindesmith et al. 2008). For example, a recent survey of the CaliciNet program from 2009 to 2013 found that GII.4 was implicated in 72% of nearly 4,000 reported NoV outbreaks in the United States (Vega et al. 2014). This genotype has been found to circulate and evolve rapidly after periods of stasis (a phenomenon called epochal evolution) such that different pandemic and epidemic strains emerge every few years. These have included Camberwell (1994), US 95/96 (1995–1996), Farmington Hills (2002), GII.4b (2002), Hunter (2004), Sakai (2006), Minerva (2006), New Orleans (2009), and Sydney (2012). Interested readers are referred to White et al. (2014) and Vinjé (2015) for additional discussion of this subject.

Multiple mechanisms contribute to the rapid evolution of GII.4 NoVs. First, RNA viruses have very high mutation rates because of a lack of proofreading mechanisms during their replication, and GII.4 appears to have higher replication and mutation rates relative to other human NoVs (Bull et al. 2010). Because GII.4 strains cause acute symptoms but rarely death, and because they affect such a large proportion of the population, the viruses may be under selective pressure from widespread host immune responses. As a result, human NoV GII.4 strains change their receptor binding and antigenic profiles over time (Debbink et al. 2012; Lindesmith et al. 2008, 2012, 2013) in an effort to escape herd immunity. This results in a change in capsid structure that affects host cell binding profiles (HBGA-mediated and perhaps others) and antigenic sites, leading to a new pool of susceptible individuals. This concept of viral capsid mutation to evade host immune response is termed antigenic drift and is supported by studies noting changing antigenic profiles of the recently circulating GII.4 New Orleans (Lindesmith et al. 2013) and Sydney (Debbink et al. 2013) strains. Such changes in antigenic profiles pose challenges for the creation of vaccines and the development of detection assays with universal recognition ligands.

Another aspect of human NoV evolution is the occurrence of recombinant strains, as these viruses have been found to undergo spontaneous recombination at different genome locations including ORF1 (Waters et al. 2007), the ORF1/2 junction (Bull et al. 2005, Eden et al. 2013), the ORF2/3 junction (Eden et al. 2013), and ORF2 (Eden et al. 2013, Rohayem et al. 2005). Investigators have recently reported novel recombinants of multiple genotypes. For example, an emerging GII.12 strain is a likely recombinant (with a GII.g ORF1 and GII.12 ORF2) (Giammanco et al. 2012, Takanashi et al. 2011) that interestingly did not bind synthetic HBGAs yet infected humans (Takanashi et al. 2011). This same strain had a rate of evolution comparable to a previously reported rate for GII.4 (Giammanco et al. 2012).

Researchers have recently used patients with chronic human NoV infections as models to observe intrahost evolution of the viruses. Bull et al. (2012) performed pyrosequencing on stool samples obtained from a chronically infected infant, finding notable heterogeneity over time to the extent that the virus in fecal samples after 288 days had become genetically distinct from the initial sampling points. Another study on chronically infected patients found multiple instances in which antigenic site mutations occurred during the infection (Siebenga et al. 2008). In short, human NoV GII.4 evolution occurs by multiple means and results in the emergence of new strains with new host susceptibility patterns, allowing the virus to continue to maintain itself even in the face of widespread population immunity (Bull et al. 2010, Donaldson et al. 2008, White 2014).

5. DETECTION OF HUMAN NOROVIRUS IN ENVIRONMENTAL AND FOOD SAMPLES

5.1. General Overview

Despite extensive efforts, development of robust methods to detect viral contamination in foods and environmental samples remains challenging. Because the numbers of virus particles present in contaminated foods are usually low and no universal or rapid culture-based methods are available, enrichment is not possible. Consequently, the viruses must be concentrated and purified from complex sample matrices prior to detection, with molecular amplification (reverse transcription qPCR or RT-qPCR) being the detection method of choice. Sequence-based determination of amplicon identity is ideal for amplicon confirmation. The major steps required for the detection of viruses in foods can be designated as follows: (*a*) virus concentration and purification, (*b*) nucleic acid extraction, (*c*) detection, and (*d*) confirmation. A full description of these methods is well beyond the scope of this review. Recent efforts at international standardization are summarized in **Figure 2**, although there are many other methods reported in the literature (ISO 2013). The interested reader is referred to more comprehensive reviews (Bosch et al. 2008, Butot et al. 2014, Knight et al. 2013, Maurer 2011, Vinjé 2015). Here we discuss two recalcitrant problems associated with the detection of NoV in complex sample matrices: (*a*) interpretation of presumptively positive samples and (*b*) the infectivity dilemma.

5.2. Detection and Confirmation—The Interpretation Dilemma?

Owing to their very low detection limits (theoretically, a single genome copy per RT-qPCR reaction), molecular amplification approaches are the methods of choice for detecting human NoV in food and environmental samples in which the concentration of viruses is usually quite low. Until the mid-2000s, sample concentrates were processed for viral RNA isolation, followed by detection by RT-PCR and confirmation of amplicon identity using nucleic acid hybridization. In the middle of the last decade, RT-PCR was replaced almost exclusively by RT-qPCR, a method that incorporates a fluorescently labeled probe or specifically intercalating fluorescent dye in the reaction mix, theoretically allowing one to bypass time-consuming DNA hybridization steps. This method currently remains the gold standard.

Incorporation of a hybridization step during amplification can be problematic when low template concentrations are present. First, matrix-associated inhibition persists in many food and environmental samples and can impact the efficiency of nucleic acid extraction and RT-qPCR (Rutjes et al. 2006, Schrader et al. 2012). Such inhibition is usually identified using internal amplification controls, but these can interfere with the efficiency of amplification of the target (Hoorfar et al. 2004). When inhibition occurs, the usual solution is sample dilution. Unfortunately, this then reduces the analytical sensitivity of the assay.

Another issue is interpretation of results from samples producing high cycle threshold (Ct) values by RT-qPCR. Numerous studies have employed RT-qPCR to detect viruses in various types of naturally contaminated sample matrices, and a snapshot of these studies is provided in **Table 1**. In most cases, some sort of amplicon confirmation step was used, ranging from cloning and sequencing to double amplifications to hybridization. The most reliable of these is DNA sequencing, but rarely is enough amplicon obtained from naturally contaminated samples to successfully acquire sequences, especially when Ct values exceed 35. Unfortunately, low levels of viral contamination (and associated high Ct values) are the rule rather than the exception for these sample types.

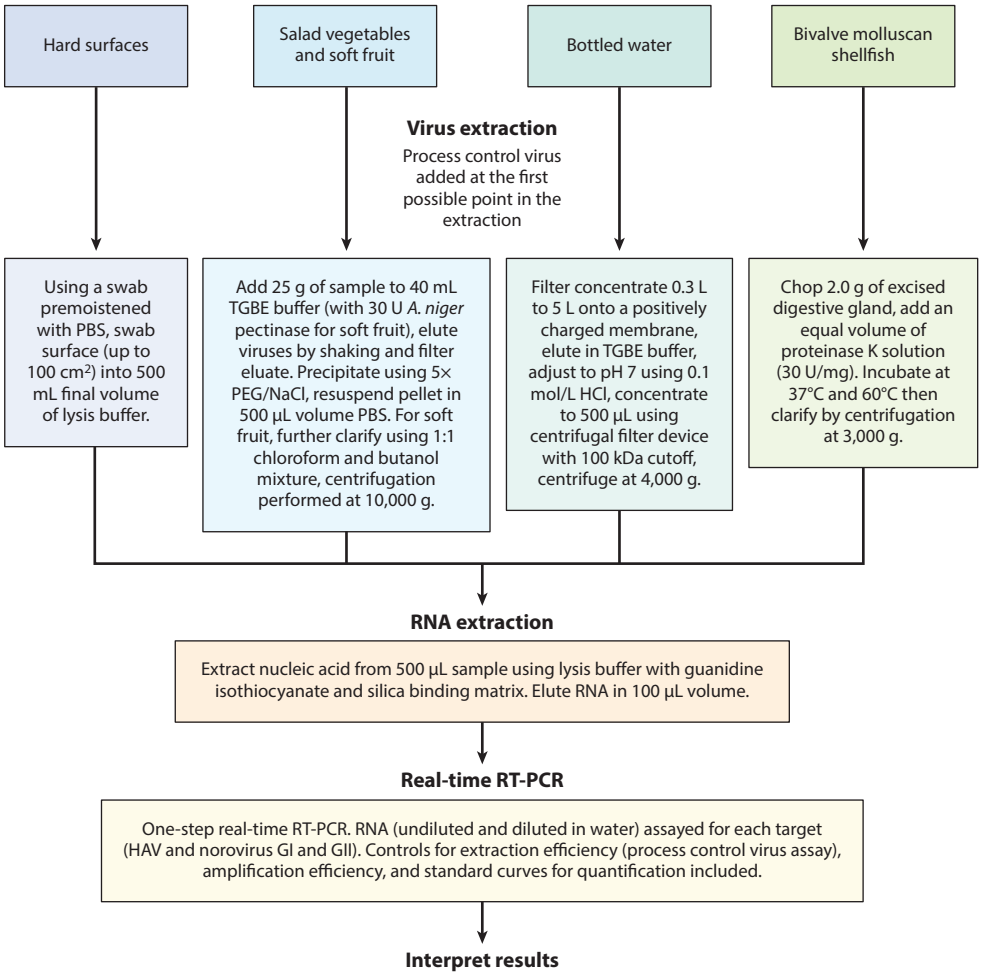

Figure 2

Diagram of the International Organization for Standardization (ISO) method for the determination of HAV and norovirus in food and water samples. Image adapted from ISO/TS 15216-1, Annex A (figure A.1). Abbreviations: *A. niger*, *Aspergillus niger*; HAV, hepatitis A virus; PBS, phosphate-buffered saline; PEG, polyethylene glycol; TGBE, tris/glycine/beef extract.

Thus, in many cases, definitive confirmation of amplicon identity may not be possible. This means that the analyst is faced with the conundrum of wrongly interpreting sample positivity, leading to false positive or false negative reporting. At this point in time, it is probably prudent to interpret such samples as presumptively positive but to also keep in mind that sample negativity is impacted by residual RT-qPCR inhibition (and potential sample dilution), competition with internal amplification controls, and sampling methodology. Even a negative result does not assure the analyst of the absence of viral contamination. Unfortunately, these interpretation dilemmas remain unaddressed. As more food and environmental samples are screened, it is increasingly important for researchers to come to a consensus on these data interpretation issues; otherwise, they have the chance of under- or overestimating the prevalence of virus contamination, wrongly predicting its risk, or both.

Table 1 Studies highlighting issues in positive RT-qPCR interpretation

Sample type	Controls and confirmation	Issues	Reference
Leafy greens	Second PCR targeting a different region and attempted sequencing of amplicon	Of 275 samples total, 148 (54%) were positive for HuNoV by RT-qPCR. Only 40 samples (15%) produced a band of expected size for the second amplification. Of these, only 16 (6%) could be sequenced to confirm HuNoV RNA.	Mattison et al. 2010
Raspberries, cherry tomatoes, strawberries, and fruit salad	IAC included; attempted sequencing of amplicon	Positive Ct values were >37. Not a single positive could be confirmed by sequencing.	Stals et al. 2011
Environmental (catering kitchens and restrooms)	Nested PCR; attempted sequencing amplicon	Samples with Cts >40 could not always be confirmed by sequencing.	Boxman et al. 2011
Raw and treated sewage water	Southern hybridization	Positive RT-qPCR and hybridization results were not always correlated.	van den Berg et al. 2005
Shellfish	Dilution, hybridization, and attempted sequencing of amplicon	Sequencing was not possible. Sensitivities differed for hybridization compared to RT-qPCR between GI and GII genogroups of NoV. Inhibition was seen in 27% of positive samples.	Loisy et al. 2005
Shellfish	IAC and sequencing	Most positive virus samples had Cts >45 (of 50 cycles). Of 15 positive samples, only 5 could be sequenced.	DePaola et al. 2010
NA	NA	This broad review focuses on molecular methods in food microbiology.	Ceuppens et al. 2014

Abbreviations: Ct, cycle threshold; HuNoV, human norovirus; IAC, internal amplification control; NA, not applicable; NoV, norovirus; PCR, polymerase chain reaction; RT-qPCR, real-time quantitative PCR.

5.3. The Infectivity Dilemma

Methods designed to detect viral nucleic acids are unable to distinguish between infectious and noninfectious viruses, the latter of which might consist of defective virus particles and mutated or degraded viral RNA. This is because RT-qPCR relies on the amplification and detection of small nucleic acid sequences (approximately 100–200 nucleotides) that can originate from the complete infectious viral genome, defective virus particles containing intact or partially intact genomes, and/or degraded RNA derived from inactivated particles. Furthermore, the scientific community has unequivocally demonstrated that the RNA associated with inactivated viruses remains detectable by nucleic acid amplification long after viral infectivity has been lost (Richards 1999). In short, in the absence of a culture method or a suitable marker for infectivity, it is impossible to confirm that the detection of human NoVs by RT-qPCR is indicative of infectious virus, as RT-qPCR tends to overestimate the actual amount of infectious virus. There are two major reasons why food scientists need reliable means to discriminate human NoVs infectivity status: (*a*) to identify products that actually pose a risk to human health and (*b*) to support studies of virus inactivation, including characterization of the efficacy of both traditional and novel control measures.

Researchers have investigated alternative methods for predicting virus infectivity based on molecular amplification approaches. These methods fall into two major classifications: (*a*) those based on examining the stability of the virus genome and (*b*) those based on examining capsid integrity. Examples of these approaches are provided in **Table 2**. The latter approach is the most

popular, and one commonly used method is to precede RT-qPCR with enzymatic (RNase with or without prior proteinase K) treatment. Sometimes called virolysis, the idea is that destruction of the viral capsid will result in release of viral RNA, which will be degraded by RNase. If the capsid is only partially degraded, then a prior proteinase K digestion should finish the process, releasing the viral RNA so that it is susceptible to RNase. A second major approach is the so-called integrated method, in that it is designed to measure the affinity of virus particles to receptors involved in cellular attachment or to other virus-binding ligands. Commonly used ligands are antibodies, carbohydrates (HBGAs), or negatively charged (cationic) magnetic particles. In these cases, infectivity discrimination assumes that if the viral capsid is not intact or denatured, it will not be able to bind to the receptors.

Some inactivation methods target the viral nucleic acid. In these cases, detection of small genomic target fragments using RT-qPCR may not indicate the presence of infectious virus for two reasons. First, naked viral RNA can persist long after a viral capsid has been destroyed. Second, even though a single strand break occurring anywhere in the viral genome will render a virus particle noninfectious, the same break cannot be detected by RT-qPCR if the target region for amplification remains intact. To account for this problem, some researchers have suggested that measurement of the overall integrity of genomic RNA could provide a useful marker for infective particles. This has been accomplished by techniques using multiple amplifications (Pecson et al. 2011), amplification of near-full-length genomes (Kostela et al. 2008), or a combination in which long-range reverse transcription is followed by more efficient small-fragment RT-qPCR (Wolf et al. 2009).

One method that accounts for both capsid and genome integrity is the use of nucleic acid intercalating agents such as propidium monoazide (Escudero-Abarca et al. 2014, Kim & Ko 2012, Parshionikar et al. 2010). Theoretically, these compounds cannot penetrate intact capsids but can penetrate damaged or destroyed capsids. Once penetrated, the photoinducible azide group on these molecules covalently cross-links the RNA, producing stable monoadducts that cannot be amplified by PCR.

Each of the methods proposed to discriminate the infectivity status of human NoVs using nucleic acid amplification has its own advantages and disadvantages, and the reader is referred to the comprehensive review of Knight et al. (2013) for more details. There is great debate among scientists working in this field as to which is the best method. When looking at the data collectively as applied to surrogate viruses for which infectivity and nucleic acid amplification are compared, preceding RT-qPCR with enzymatic pretreatments or ligand binding (capsid integrity methods) provides better, but still not complete, agreement with virus infectivity. Methods to assess genome integrity and the use of nucleic acid intercalating agents are difficult to optimize and frequently inefficient. Also, data on method performance frequently differ as a function of inactivation strategy. To date, no one method has been demonstrated to accurately discern infectious from noninfectious viral particles in the absence of an in vitro human NoV cultivation method.

6. CONCLUSIONS

In all, human NoVs remain a significant challenge. Elements of the basic biology and replication mechanism of human NoVs remain to be elucidated. Although many in vitro and in vivo cultivation methods have been attempted, humans remain the only host for virus propagation. As such, researchers have been forced to rely on data produced using cultivable surrogates, but each of these has its own advantages and disadvantages. Human challenge studies can also be conducted, but they are expensive, time consuming, and infrequent. Rapid strain divergence and antigenic drift

Table 2 Studies regarding in vitro estimation of human norovirus infectivity using RT-qPCR

Method	Viruses tested	Treatment(s)	Results	Reference(s)
Long-range genome amplification	MS2, HuNoV GI.1 and GII.4, MNV, PV1	UV, heat	Method generally overestimated viral load by 0.5 to as much as 4 $\log_{10}$ but usually performed better than traditional RT-qPCR. For UV, method design had to account for different genomic region susceptibility. Amplification tended to be inefficient. Does not account for capsid integrity.	Kostela et al. 2008, Pecson et al. 2011, Shin & Sobsey 2003, Wolf et al. 2009
RNase and Proteinase K pretreatment followed by RT-PCR	MS2, MNV, HAV, PV1, FCV, HuNoV GII	UV, hypochlorite, heat, singlet oxygen, HPP	Method generally overestimated viral load by 2–3 $\log_{10}$, sometimes performing better than traditional RT-qPCR. One study gave negative end point results, but low initial loads of virus were used ($\sim 10^3$ $\log_{10}$). Generally does not account for genome integrity.	Diez-Valcarce et al. 2011, Nuanualsuwan & Cliver 2002, Pecson et al. 2009, Tang et al. 2010
RNase pretreatment with RT-qPCR	FCV, MNV, human adenovirus, HuNoV GI.1	Hypochlorite, heat, alcohols, quaternary ammonium compounds, chlorine dioxide, oregano extract	Method correlates to infectivity over a narrow disinfection range for hypochlorite (low available chlorine concentrations) and heat initially but overestimates viral load in most cases by 1–>3 $\log_{10}$. A human feeding study found this method overestimated viral persistence in groundwater by nearly three years. Mathematical modeling was applied to make the estimation fairly accurate in one case. Generally does not account for genome integrity.	de Abreu Corrêa et al. 2012; Gilling et al. 2014; Nowak et al. 2011a,b; Seitz et al. 2011; Topping et al. 2009
Cell binding (RAW 264.7 or Caco-2) or receptor binding (ganglioside GD1a or PGM) followed by RT-qPCR	MNV; HuNoV GI.1, GI.8, and GII.4	Heat, HPP, UV, hydrogen peroxide, grape seed extract	Some evidence that PGM binds preferentially to intact particles. Collectively, the method overestimated viral load by 1–4.5 $\log_{10}$ but usually performed better than traditional RT-qPCR. Generally does not account for genome integrity.	Dancho et al. 2012; Li et al. 2011, 2012a; Tang et al. 2010
Pretreatment with nucleic acid intercalating agents (PMA) followed by RT-qPCR	HAV, HuNoV GII.2, MS2, MNV	Heat	Two studies compared PMA-RT-qPCR to RNase-RT-qPCR. The former method appeared to perform equivalently or better than the latter and certainly better than RT-qPCR alone. However, the method is difficult to optimize, and results appear to vary by virus.	Escudero-Abarca et al. 2014, Kim & Ko 2012, Parshionikar et al. 2010, Sánchez et al. 2012
Hydrazide binding followed by RT-qPCR	Astrovirus serotype 4, HuNoV GII.4	Hypochlorite	Method overestimated astrovirus load by about 1–2 $\log_{10}$ compared to plaque assay. Only relevant for disinfection approaches in which oxidative damage occurs.	Sano et al. 2010
Proteinase K and RNase pretreatment followed by NASBA	FCV, HuNoV GII	Heat	Enzymatic treatment removed all positive signals for HuNoV that had been seen without treatment. No comparison to surrogate to demonstrate method was made, however.	Lamhoujeb et al. 2008

Abbreviations: FCV, feline calicivirus; HAV, hepatitis A virus; HPP, high-pressure processing; HuNoV, human norovirus; MNV, murine norovirus; MS2, bacteriophage MS2; NASBA, nucleic acid sequence–based amplification; PGM, porcine gastric mucin; PMA, propidium monoazide; PV1, poliovirus I; RT-qPCR, real-time quantitative polymerase chain reaction; UV, ultraviolet light.

of the GII.4 genotype presents an additional set of challenges, particularly in vaccine development and detection of emerging strains. Although our understanding of disease burden and attribution is improving, outbreaks are still vastly underreported, and adequately addressing the important role of the food handler remains a recalcitrant issue. In the absence of reliable detection methods for viruses in foods, it is difficult to attribute specific foods to outbreaks or to better understand the prevalence of naturally occurring contamination. In addition to its existing features as a near-perfect foodborne pathogen, we now need to add another: our inability to really understand human NoVs because they are so difficult to study.

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