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Predicting human norovirus infectivity - Recent advances and continued challenges

Clyde S. Manuel[a,1], Matthew D. Moore[b,*], Lee-Ann Jaykus[a]

[a] Department of Food, Bioprocessing, and Nutrition Sciences, North Carolina State University, Raleigh, NC, 27695, USA
[b] Department of Food Science, University of Massachusetts, Amherst, MA, 01003, USA

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ABSTRACT

Human norovirus is the leading cause of foodborne illness globally, imposing a considerable public health and economic burden. Historically, one of the major obstacles to the study of human noroviruses has been the lack of an *in vitro* cultivation system. In addition to hindering elucidation of viral pathogenesis, research efforts have been limited by the inability to discriminate infectious from non-infectious viral particles. Two recent breakthrough human norovirus *in vitro* cultivation system systems have been reported, but in their current state, may be unsuitable for routine detection or study of human noroviruses in the food and water sciences. More accessible alternative techniques utilizing molecular assays, animal models, and surrogate virus systems for prediction of human norovirus infectivity have been presented. The purpose of this review is to present the multiple recent techniques used to assess human norovirus infectivity, including recently described human norovirus *in vitro* cultivation systems, cultivable surrogate viruses, animal models, and alternative molecular techniques, and discuss their advantages and disadvantages in the context of determining human norovirus infectivity.

1. Introduction

Human norovirus is the leading cause of foodborne gastroenteritis worldwide and is estimated to be responsible for 125 million foodborne illnesses annually on a global basis (Kirk et al., 2015). In the United States, the virus is estimated to cause 21 million illnesses annually (Hall et al., 2013), of which 5.5 million are associated with food (Scallan et al., 2011). Symptoms usually include vomiting, diarrhea, and abdominal pain, typically lasting 24–48 h after onset. Mortality rates are low and severe complications arising from illness are rare. Nonetheless, norovirus is estimated to cause 56,000–71,000 hospitalizations and 570–800 deaths every year in the U.S. by virtue of its high disease prevalence (Hall et al., 2013). The cost to healthcare and lost productivity associated with foodborne norovirus in the U.S. has been estimated at over $2 billion annually (Hoffmann et al., 2012; Scharff, 2012). This number, however, accounts for only about one-quarter of all cases, as the majority of norovirus transmission is not foodborne (Hoffmann et al., 2012; Scharff, 2012).[2]

Numerous factors contribute to norovirus being such a prevalent pathogen. First, it has a low infectious dose (< 20 viral particles) (Teunis et al., 2008). Second, the virus is shed at high concentrations (10^6 to 10^9 particles per gram of stool) during symptoms, and may potentially be shed well after symptoms have passed (4–8 weeks) (Atmar et al., 2008). Third, the virus is released in high numbers in the vomitus of infected individuals (Atmar et al., 2014; Caul, 1994; Greenberg et al., 1979), and is aerosolized during vomiting events (Repp and Keene, 2012; Tung-Thompson et al., 2015), which contributes to its spread. Finally, norovirus is able to persist once deposited onto environmental surfaces, as it can potentially remain infectious for several weeks (Lopman et al., 2012) and may be difficult to inactivate due to the virus's extreme resistance to many sanitizers and disinfectants used at manufacturer recommended or regulated concentrations and/or contact times (Escudero et al., 2012; Tung et al., 2013).

Historically, norovirus has been difficult to study due to a lack of a readily available animal cell culture model. This lack of a cell culture model has forced researchers to rely on cultivable surrogate viruses, which do not always behave similarly to human norovirus (Richards, 2012). Multiple surrogates have been identified that allow for evaluation of infectivity using plaque assays and 50% tissue culture infectious dose ($TCID_{50}$) assays. Efforts to culture human norovirus have seen tremendous advances in recent years, yet these methods may not yet be ready for routine use (discussed below). Detection of human norovirus

* Corresponding author.
E-mail address: mdmoore@umass.edu (M.D. Moore).
[1] Current address: Diversey Inc., 2415 Cascade Pointe Blvd, Charlotte NC, 28208.
[2] RT-qPCR/PCR: reverse transcriptase quantitative polymerase chain reaction; MNV: murine norovirus; TV: Tulane virus; FCV: feline calicivirus; HBGA: histo-blood group antigen.

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is most often performed using molecular methods that amplify a portion of its RNA genome such as RT-qPCR. However, these techniques are not able to discriminate between infectious and non-infectious viruses, largely because amplification of all RNA will occur, including free unencapsidated RNA, partially damaged/degraded RNA, RNA enclosed in damaged capsids, and RNA with lethal mutations. These uncertainties with respect to the integrity of the virus particle create complications in RT-qPCR data interpretation when applied to prevalence studies and to validate the efficacy of candidate norovirus inactivation strategies. As knowledge of the importance of norovirus to foodborne disease has increased, the ramifications of this "infectivity dilemma" have become more widely recognized. The purpose of this review is to highlight recent progress in both culture-based and molecular techniques for discriminating infectious human norovirus particles.

2. Currently available models for determining norovirus infectivity

2.1. Recent advances in development of a human norovirus in vitro cultivation system

Since the first characterization of human norovirus in 1972 (Kapikian et al., 1972), development of a cell culture model for its replication has been one of the most sought after goals in the field of food virology. Numerous laboratories have spent countless hours and resources in an attempt to culture human norovirus in a wide variety of cell lines, all without success (Duizer et al., 2004; Moore et al., 2015b). In 2007, researchers reported successful norovirus replication in two separate human epithelial cell lines using three-dimensional organoids grown in a rotating bioreactor system (Straub et al., 2007, 2011). However, attempts to replicate those studies failed (Papafragkou et al., 2014; Takanashi et al., 2014) and the system was abandoned.

Several recent breakthroughs in attempts to cultivate human norovirus *in vitro* have been reported. The first findings were published in by Jones et al. (2014). Motivated by observations that chimpanzees infected with human norovirus can produce capsid protein-positive B-cells (Bok et al., 2011), and from observations that certain enteric bacteria can possess histo-blood group antigens (HBGAs) (Miura et al., 2013), the putative receptors for human norovirus infection (Hutson et al., 2002), Jones et al. (2014) pursued the development of an *in vitro* cultivation system. Specifically, they achieved a 600-fold increase in viral genome copy number in a B-cell line (BJAB) challenged with a human norovirus strain when enteric bacteria were included. Specifically, this successful *in vitro* human norovirus replication required HBGA type H in free form or derived from HBGA-expressing enteric bacteria (*Enterobacter cloacae*). Jones et al. (2014) concluded that bacterial HBGAs may act as a co-factor for viral replication rather than human HBGAs acting as a sole receptor as previously hypothesized.

While the results of Jones et al. (2014) are promising, the routine use of the B-cell cultivation system faces several hurdles. First, co-incubating enteric bacteria (*E. cloacae*) with human norovirus on cell monolayers can result in inadvertent bacterial contamination of cell lines if aseptic practices are not strictly maintained. A much more practical alternative is to use HBGAs directly in solution, but these reagents can be expensive and can suffer from quality issues due to the difficult nature of their synthesis and purification. Second, human norovirus infection in B-cells does not produce a visible cytopathic effect (Jones et al., 2014), and so quantification will still rely on RT-qPCR. A protocol for this approach has also been published (Jones et al., 2015), and it has been replicated in the study on the antiviral effects of a human norovirus polymerase inhibitor (Kolawole et al., 2016).

More recently, researchers have successfully cultivated several strains of GII.4 and GII.3 human norovirus in stem cell-derived human intestinal enteroids (Ettayebi et al., 2016). In this system, enteroid monolayers are created through the differentiation of stem cells derived from the duodenum, jejunum, or ileum. Treatment of enteroid monolayers with bile acids was required for GII.3 strain replication, whereas this treatment was not required but enhanced replication of GII.4 strains. It remains to be seen what individual component of bile is responsible for this phenomenon. Strengths of this system include its ability to support replication of multiple strains of human norovirus, the ability to display cytopathic effects, and its consistency with epidemiological observations regarding host HBGA patterns and infection. The ability to observe a cytopathic effect would allow for application of a $TCID_{50}$ assay in study of antiviral agents. A major drawback of the enteroid system is the length of time and high costs associated with establishing and maintaining this system, which essentially renders the system impractical for routine food/water testing. Both this enteroid model and the B cell model discussed above represent exciting breakthroughs in the field of human norovirus research and will no doubt be critical for bettering our understanding of these viruses; however the need for alternative *in vitro* molecular methods to discriminate infectious human norovirus particles will likely persist.

2.2. Animal models for human norovirus

Numerous animal models have also been investigated for their ability to support human norovirus replication. When challenged with GII.4 norovirus, gnotobiotic piglets display many symptoms consistent with viral infection, including mild diarrhea, viral shedding, cytopathic lesions in intestinal biopsies, and seroconversion indicative of an immune response (Cheetham et al., 2006). Recently, this gnotobiotic piglet model was used to assess the efficacy of high pressure processing (HPP) for inactivating a GII.4 norovirus strain seeded in oyster homogenates (Lou et al., 2015). The authors found that HPP conditions of 350 MPa for 2 min at 0 °C were able to completely abolish all cytopathic effects of the virus, indicating these HPP conditions to be sufficient for complete inactivation of human norovirus (Lou et al., 2015). While a novel application, this model has its limitations restricting routine use, including high cost (one litter of piglets required for a study is estimated to cost approximately $25,000) and the need for specialized animal facilities, thus limiting the model's scalability (Lou et al., 2015).

The presence of human norovirus antibodies in several non-human primate species (Jiang et al., 2004) has prompted researchers to investigate this animal model. When challenged with human norovirus strains, both rhesus macaques (Rockx et al., 2005) and pigtail macaques (Subekti et al., 2002) were able to support infections for some cases, albeit without symptoms. When injected intravenously with filtrate from a human norovirus positive stool sample, chimpanzees were shown to exhibit many signs of infection, including production of viral antigens and viral shedding (Bok et al., 2011). However, the use of non-human primates is subject to the same limitations as the gnotobiotic pig model, with the additional problem of ethical implications (Goodman and Check, 2002). Additionally, the National Institutes of Health (NIH) recently announced the cessation of funding for research involving the use of chimpanzees (Kaiser, 2015).

A mouse model was also recently reported in which replication of a GII.4 human norovirus strain in immune deficient BALB/c mice was observed (Taube et al., 2013). Interestingly, viral replication was not dependent on the "humanized" (mice grafted with human hematopoietic stem cells) status of the mice, but rather the fact that they were immune deficient, as wild type mice did not support viral replication (Taube et al., 2013). While use of a mouse model might be more appealing, the reliance on immune deficient mice, the need for intraperitoneal challenge, and the lack of a visible disease state in infected animals complicates its widespread applicability.

2.3. Cultivable norovirus surrogates

The historical absence of a reliable cell culture or animal model for human norovirus propagation has led to the widespread use of