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Review article

Applications of Surrogates and Culture Systems in Norovirus Research

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Abstract

Norovirus (NoV) is a leading cause of acute gastroenteritis and foodborne illness across all age groups. Since its discovery in 1968, research has been hindered by the lack of a robust in vitro culture system, prompting reliance on surrogate caliciviruses. Recent breakthroughs in NoV culture models-including human intestinal organoids and zebrafish larvae-now offer physiologically relevant platforms to study viral entry, replication, and therapeutic intervention.

Keywords: Norovirus; Surrogate models; Culture systems

Introduction

Norovirus (NoV), a non-enveloped, positive-sense single-stranded RNA virus of the Caliciviridae family, has a 7.5-7.8 kb genome organized into three ORFs (Fig.1). ORF1 encodes six non-structural proteins, including RdRp; ORF2 encodes the major capsid protein VP1 that assembles into T=3 icosahedral virions for receptor binding and antigen display; ORF3 encodes minor capsid protein VP2 that stabilizes virions and facilitates genome delivery. Replication initiates within replication complexes, generating a negative-strand intermediate followed by full-length genomic and ORF 2-3-spanning subgenomic RNAs [1-5].

Norovirus is the leading etiologic agent of non-bacterial epidemic gastroenteritis. Human norovirus (HuNoV) is estimated to cause 685 million infections and 200,000 deaths annually-50,000 of them in children-while imposing US4.2 billion in direct health-care expenditures and US60 billion in broader societal costs every year [6, 7]. Although HuNoV gastroenteritis is usually self-limiting, immunocompromised or immunodeficient populations-infants, the elderly, and organ-transplant recipients-frequently progress to chronic infection with severe complications. The absence of a robust in vitro culture system has severely constrained elucidation of the viral life-cycle at the molecular level and, consequently, the development of targeted antiviral therapeutics and vaccines; to date, neither specific antivirals nor licensed vaccines are available.

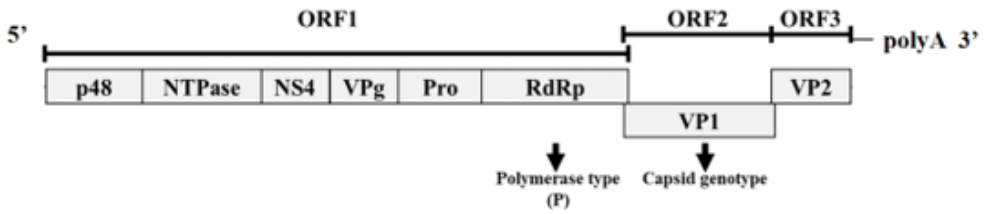

Figure 1: Genomic organization of norovirus.

HuNoV Research Substitutes

Surrogate Viruses

Despite its high infectivity and ability to replicate to extremely high titers in the human gut, researchers have extensively explored sensitive and permissive cell lines for HuNoV, such as human gastrointestinal epithelial cell lines, but all attempts have failed [8-11] This failure is attributed to the substantial differences between laboratory-cultured cell lines and the native human intestinal environment, which prevent the successful cultivation and maintenance of HuNoV in vitro. Consequently, researchers have long relied on surrogate viruses for studies, typically noroviruses from non-human hosts or other calicivirus species, such as mouse norovirus (MNV) [11], feline calicivirus (FCV) [12], Porcine Sapporo virus (PSV) [13], and Turanavirus (TV) [14]. These surrogate viruses can replicate stably in appropriate cell lines, partially fulfilling experimental requirements.

MNV can persistently replicate in the mouse macrophage cell line (RAW264.7), which is widely used to investigate norovirus infection mechanisms, antiviral therapies, and host immune responses [15, 16]. However, the MNV genome contains an additional open reading frame (ORF4) compared to HuNoV, meaning findings from MNV studies cannot be directly extrapolated to HuNoV. For example, CD300lf is an essential protein receptor for MNV infection in mice, but human CD300lf is not a receptor for HuNoV [17]. This indicates that the infection mechanisms of HuNoV and MNV are distinct.

FCV and PSV are commonly used to evaluate viral resistance and are of significant value for environmental disinfection products and pathogen control in food enterprises [18, 19]. The TV genome structure is highly similar to that of HuNoV and shares the same host cell receptor-histo-blood group antigen (HBGA)-with HuNoV, facilitating research on virus-associated receptors and co-factors [14].

Reverse Genetics and Virus-Like Particles

Since 2002, researchers have attempted to generate infectious noroviruses and replicons using reverse genetics, achieving their in vitro propagation. This research direction has greatly advanced studies on the norovirus replication cycle and its pathogenic mechanisms [20]. Katayama and Oliveira developed infectious recombinant HuNoV and replicons in 2014 and 2016, respectively, but these were not widely adopted by other p due to their low efficiency [21, 22]. Reverse genetics systems cannot achieve indefinite replication cycles, making the improvement of viral rescue efficiency within finite replication cycles a key focus for future research.

Additionally, researchers have produced HuNoV virus-like particles (VLPs) through molecular cloning. These VLPs possess receptor-binding domains and antigenicity similar to the authentic virus, allowing them to partially substitute for HuNoV in studies of antigenic drift and antibody immune responses [23].

In Vitro / Cell Culture Models

B Cell Lines

In 2014, Jones et al. first reported in Science that HuNoV GII.4 could replicate in the human B lymphoma cell line BJAB [24]. Subsequent studies by the same group demonstrated that, with the assistance of exogenous HBGA, only GII.4 and GII.6 genotypes could replicate in BJAB cells, with a 10-50-fold increase in viral load. However, the progeny virus failed to propagate to subsequent cell passages [25]. Successful HuNoV infection of primary B cells was also achieved, showing a replication level comparable to that in BJAB cells [26]. Despite being the first cell line reported to support HuNoV replication a decade ago, only a limited number of laboratories have successfully reproduced this system, and the underlying cause of its poor reproducibility remains unclear [27, 28].

Human Intestinal Organoids

In 2016, Ettayebi et al. published the first study in Science using human intestinal organoids (HIOs) to cultivate HuNoV [29]. Unlike B cells, HuNoV replication in HIOs does not require exogenous HBGA and reaches approximately a 3 $\log_{10}$ increase, with successful passage for up to four generations. Infection of GI.1, GII.3, and GII.17 strains requires bile, whereas GII.4 infection does not, indicating genotype-dependent cell tropism. The Estes laboratory extensively distributed and promoted this system, enabling many groups to reconstruct it successfully. Over the past eight years, HIOs have become a widely adopted platform for HuNoV research, contributing to breakthroughs in understanding viral pathogenesis [30, 31], antiviral drug development [32] viral environmental stability [33]

and inactivation [34] as well as neutralizing antibody responses [35]. Nevertheless, the high cost and technical complexity of the system have driven efforts to develop more accessible models.

Salivary Gland Cell Lines

Ghosh et al. reported in Nature that GII.4 HuNoV can replicate continuously in SV40-immortalized human salivary gland cells, achieving a 3 log10 increase and being successfully passaged four times [36]. However, this finding has not yet been independently reproduced in other laboratories, and the specific cellular targets and cofactors involved in HuNoV replication in salivary cells remain to be elucidated.

In summary, every new HuNoV cell culture model has been published in high-impact journals such as Science or Nature, underscoring the critical and urgent need to establish a robust and reproducible HuNoV culture system.

In Vivo / Animal Models

Mammalian Models

Extensive efforts have been made to explore various animal species as potential models for HuNoV infection and propagation. To date, chimpanzees, rhesus macaques, gnotobiotic pigs, and cattle have been identified as susceptible hosts [37, 38]. Infected chimpanzees shed infectious viral particles in their feces, which can transmit infection to chimpanzee offspring [39]. Rhesus macaques can be orally infected with both GI and GII genogroups, and while they mount a specific immune response, viral shedding can persist for several weeks-longer than in humans, where the virus is typically cleared within one week [38]. GII.4 HuNoV has also been successfully passaged twice in gnotobiotic pigs, inducing pathological changes in intestinal epithelial cells [40]. However, these large-animal models are costly and require extensive housing facilities, limiting their utility for high-throughput antiviral screening.

In small animal studies, Taube et al. first demonstrated that GII.4 HuNoV can replicate in immunodeficient mice lacking recombination activation gene 1 or 2 and the common γ chain (Rag-γc-/-), with viral detection in multiple tissues including the intestine [41]. However, this model has several limitations. Infection was achieved via intraperitoneal injection rather than the natural oral route, and infected mice did not shed infectious viral particles. Moreover, wild-type BALB/c mice failed to show signs of infection. Intriguingly, HuNoV replication was observed in both humanized and non-humanized mice, indicating that viral replication depends on host immunodeficiency rather than the presence of human immune cells. This model has since been used to evaluate candidate antiviral drugs against HuNoV infection [27].

Zebrafish Model

In 2019, Van Dycke et al. introduced a smaller and more cost-effective in vivo model using zebrafish larvae for HuNoV infection studies [42, 43]. Microinjection of viral suspensions into the yolk sac of zebrafish larvae enabled replication of GI.7, GII.3, and GII.4 genotypes. The yolk sac also supported serial passaging, with GII.4 successfully propagated to the third generation. Immunohistochemistry revealed HuNoV antigens in multiple organs, including the intestine and liver. Importantly, treatment with 2′-C-methylcytidine (2-CMC) significantly reduced viral replication, demonstrating the utility of this model for antiviral drug testing.

HuNoV replication in zebrafish larvae has been independently reproduced in multiple laboratories, highlighting its potential as a versatile platform for HuNoV research [44-46]. Cuvry et al. found that HBGA-expressing bacteria had no significant impact on HuNoV replication in germ-free zebrafish larvae, suggesting that exogenous HBGA is not required [47]. Tan et al. demonstrated efficient replication and four successive passages in zebrafish embryos [48]. Kim et al. investigated host gene expression biomarkers during HuNoV infection [46], and Toh et al. used this model to study sequential HuNoV–Salmonella infections, revealing a key role of the ACOD1/IRG pathway [49]. Although the specific target cells and cofactors for HuNoV infection in zebrafish remain unidentified, viral replication has only been observed following yolk sac microinjection at the embryonic or larval stage. The lipid-rich environment of the yolk sac and bile may act as a cofactor facilitating viral entry.

Potential Intermediate Hosts

Evidence suggests that other animal species may serve as potential intermediate hosts for HuNoV. Charoen Kul et al. reported that domestic dogs could act as transmission vectors between humans and animals [50]. HuNoV genomic sequences have also been detected in bird feces [51, 52]. Villabruna et al. screened multiple host species using VLP binding assays and found that certain HuNoV genotypes could attach to the intestinal tissues of seven different animal species [53]. These findings support the hypothesis that HuNoV may circulate in a broad range of animal reservoirs. However, whether these animals possess permissive target cells and can be utilized as experimental models requires further investigation.

Discussion and Prospect

Several limitations in existing HuNoV culture systems must be urgently addressed to accelerate progress in viral research. First and foremost, HuNoV cannot be indefinitely passaged in any culture system, and all research teams rely on clinical specimens containing the virus (such as fecal suspensions) for infection studies. Therefore, once the fecal samples are exhausted, the relevant research cannot continue. Although the aforementioned systems can support viral replication, their efficiency is far inferior to that of other viral culture systems, such as the VeroE6/TMPRSS2 cell line used for the isolation and propagation of SARS-CoV-2 [92].

Secondly, there is currently a lack of high-throughput systems capable of supporting large-scale antiviral drug screening for HuNoV, such as the need to screen thousands or tens of thousands of compounds. While the current culture system is viable for antiviral screening, even screening small compound libraries (e.g., approximately 300 compounds) requires significant effort. This is primarily due to the system's near-total reliance on RT-qPCR

assays to assess HuNoV replication, as HuNoV does not induce pronounced cytopathic effects in susceptible cells or animals. Consequently, the cytotoxicity-based drug screening method used in the VeroE6 cell model for SARS-CoV-2 is not applicable. Although the zebrafish model is commonly used for screening drugs related to developmental disorders, in the antiviral field, fish embryos or larvae are sensitive to most antiviral drugs, leading to developmental abnormalities. Therefore, it is necessary to exclude potential drug effects on zebrafish growth during the research process [54].

To overcome these challenges, an efficient HuNoV culture system must be established. The first step involves identifying HuNoV's cellular receptors and co-factors, followed by the generation of transgenic cell lines and animals overexpressing these receptors to render them highly susceptible to HuNoV infection. Despite no successful reports to date, extensive global efforts are underway to discover HuNoV's receptors. Second is optimizing the culture system for large-scale, reproducible application. The vast majority of laboratories cannot meet the experimental conditions required for using large mammals such as chimpanzees, rhesus monkeys, or germ-free pigs. While the zebrafish model offers significant cost-effectiveness advantages, establishing a zebrafish research platform requires critical equipment like microinjection systems, along with specific space and facility requirements for fish housing, often discouraging many laboratories from pursuing it. Human intestinal organoids (HIOs) are not only costly but also require fresh human cells for culture. In contrast, B-cell and salivary cell lines are more economical, yet their reproducibility, establishment, and reliability must be rigorously validated. Unfortunately, only two HuNoV genotypes (GII.4 and GII.6) can be successfully replicated in B-cell lines, and only one genotype (GII.4) in salivary cell lines. Achieving infection and replication of all HuNoV genotypes in in vitro culture systems is therefore critical. Researchers must continue optimizing existing models to enhance HuNoV replication efficiency and passage stability.

Over the past decade, norovirus researchers have made significant discoveries and gained valuable insights into the molecular mechanisms of viral infection, antiviral drugs, and inactivation conditions through novel culture systems like HIO and zebrafish models. Undoubtedly, HuNoV research has advanced several steps forward, yet it remains in its infancy. Researchers now face additional hurdles, such as those mentioned above. Developing more stable and cost-effective culture models could elevate HuNoV research to new heights.

Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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