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The makings of a *good* human norovirus surrogate

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Norovirus is undoubtedly a leading cause of acute gastroenteritis. A large limitation to the study of human norovirus is the lack of consensus research using norovirus surrogates. Over two decades of research have included vast comparisons of norovirus surrogates within the Calicivirus family. A discussion on the continued use of norovirus surrogates includes use of surrogates to adequately assess environmental persistence and food preservation technologies. Choice of proper surrogate may be influenced by a myriad of issues, including ease of propagation, genetic similarities, and binding properties. While it remains impossible to routinely culture human norovirus *in vitro* the continued use of a variety of norovirus surrogates remains crucial to facilitate an understanding of norovirus in order to reduce the public health impact of the disease.

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Introduction

The question of appropriate norovirus surrogates has plagued scientists since just after Norwalk virus was discovered in Norwalk, Ohio in 1972. A 27-nm virus-like particle lacking distinctive morphology was suggested to be the etiologic agent of the gastroenteritis outbreak in an elementary school. Soon after this virus particle was identified as the prototype strain of a large group of non-cultivable viruses that remain key agents of epidemic acute gastroenteritis in humans [18•,26]. This discovery hinged not on virus passage in cell culture or on even use of animal models, but rather on the direct study of the virus particle itself using electron microscopy. For many years, electron microscopy was used to study the detection and transmission of Norwalk-like viruses, later referred to as noroviruses, which is to date the most common cause of acute gastroenteritis in the United States. Norovirus may be spread in contaminated water, food, or from person-to-person contact. While developments for norovirus study in the laboratory includes intestinal cell culture assays, novel organ systems, and human feeding trials, which all remain on the forefronts of innovative research; the needs of food processing and safety must be met through the study of norovirus surrogates.

Norovirus surrogates

As with any surrogate for process validation or research, the list of the limitations of norovirus surrogates comes easily. What norovirus surrogates cannot provide, human feeding studies with norovirus may address [30]; however, these assays require specific clinicians and vast resources and certainly cannot be used for routine process validation or for environmental monitoring. For these reasons surrogates are a necessity today. Surrogates have benefits including the wide array of data that can be generated by scientists around the globe. A limitation of the current norovirus surrogates is that they lack the structural variation that is observed within human noroviruses [7], which may in itself serve as a reason to use multiple surrogates.

A natural start in the quest for the best surrogate is with members of the Calicivirus family. The name calicivirus is derived from the Latin word calyx meaning cup, chalice, or goblet, which is appropriate as many strains have visible cup-shaped depressions [21]. The organization of the norovirus major and minor structural capsid proteins (VP1 and VP2) form somewhat around these depressions. Structure plays an important role in protein functionality as it does in virus integrity related to capsid protein interactions. This structure–function relationship is an integral part of the maintenance of norovirus stability and infectivity. Viruses from the 7 genera in *Caliciviridae* may have potential as surrogates (*Lagovirus*, *Nebovirus*, *Norovirus*, *Sapovirus*, *Vesivirus*, *Recovirus*, *Valovirus*), and several have been studied [11]. Two widely studied caliciviruses include San Miguel sea lion virus and feline calicivirus (reviewed in [17,23,30]) while more recently much research has focused on murine norovirus and Tulane virus, as described below. Other animal viruses outside of the calicivirus family have also been studied and may serve as potential surrogates; including rotavirus and porcine saprovirus, along with non-animal viruses like bacteriophage and virus-like particles (also reviewed in [17,23,30]).

Much information has been gained by the previous studies of each surrogate. Several pertinent issues addressed in this paper will focus on use of two specific norovirus

Table 1

Comparative characteristics of contemporary norovirus surrogates. Adapted from Hoelzer *et al.* [17]

	Human norovirus	Feline calicivirus (FCV)	Murine Norovirus (MNV-1)	Tulane Virus (TV)	Coliphage (MS2 as example)
Family	*Caliciviridae*	*Caliciviridae*	*Caliciviridae*	*Caliciviridae*	*Leviviridae*
Genus	*Norovirus*	*Vesivirus*	*Norovirus*	*Recovirus*	*Levivirus*
Enteric/fecal shedding	Yes	No	Yes	Yes	No/Yes
Envelope	No	No	No	No	No
Virion diameter	27–38 nm	35–39 nm	35–39 nm	35–37 nm	27 nm
Host receptor, coreceptors	HBGA, heparan sulfate	JAM-1, sialic acid	Sialic acid, glycoproteins	HBGA	F-pilus
Genome composition	(+) ss RNA	(+) ss RNA	(+) ss RNA	(+) ss RNA	(+) ss RNA
Genome size and organization	7.5 kb 3 ORF	7.5 kb 3 ORF	7.5 kb 3 ORF	6.7 kb 3 ORF	3.5 kb

surrogates often used today, murine norovirus (MNV) and Tulane virus (TV). These two viruses are optimal surrogates for their similarities to norovirus and for their relative ease of use in laboratory and industrial settings. While MNV is a true norovirus [41,19], it is not identical to human norovirus on a molecular level as MNV infects macrophages and dendritic cells [36,42]. TV was most recently isolated and characterized from rhesus macaques at the Tulane National Primate Research Center as a representative of a new genus within *Caliciviridae*, the genus *Recovirus* [10]. Fecal transmission has been demonstrated for both MNV [24] and TV [11]. For some treatments, such as high-pressure processing (HPP), human norovirus has been shown to be more resistant than the surrogates tested (reviewed in [22]). Within this situation one may use the illustration that the surrogate should meet the worst-case scenario. This leads to a multidisciplinary approach that the choice of surrogate is best defined by the research question addressed; for example, similar host receptors may be more important to attachment and persistence studies. Table 1 compares variables that are important to the choice of a surrogate and potential limitations for each surrogate.

Food preservation

In order to have an impact on public health an effective norovirus surrogate must be robust enough to withstand processing parameters. Such a public health impact could include a reduction in annual numbers of 570–800 deaths, 56 000–71 000 hospitalizations, 400 000 emergency department visits, 1.7–1.9 million outpatient visits, and 19–21 million total illnesses of norovirus [12••]. Processing parameters include stability to pH, heat and environmental pressures. Effects of low pH will impact how a virus maintains stability in foods and through the low stomach pH in order to target infection within intestinal cells. Both MNV and TV are more readily inactivated at alkaline pH; however, inactivation is limited even at pH 10 for MNV whereas TV was more sensitive to pH [14•]. The greatest effects of pH were observed on the respiratory virus FCV, a previous commonly used norovirus surrogate. FCV is not an optimum surrogate due to its instability over a wide pH range particularly at acidic and alkaline pH [3,38]. Virus-like particles (VLPs) are only composed of viral capsid proteins and may show how virus capsid integrity is affected by treatments. Norwalk VLPs remained intact at an acidic pH of 2.5, but disassembled at pH > 9.0 as determined by mass spectrometry [32]. It should be noted that while the VLPs remained intact at low pH the viral infectivity may be altered by subtle changes within the receptor proteins within the capsid. This information cannot be garnered from experiments with VLPs; however this type of information may be gleamed from comparative research with other norovirus surrogates.

Heat is a commonly used food preservation technology and also serves as an important control for virus contamination during proper composting within environmental matrices. In composting of dairy manure and plant materials, both MNV and TV were inactivated beyond the limit of detection at 75°C [14•]. FCV showed similar thermal inactivation characteristics [3,8]. These results are similar to those observed with human norovirus [6], which was found to maintain infectivity after 30 min at 60°C, as were MNV and TV [14•]. All three surrogates were found to have three *D*-values of ~1 min at 72°C [38]. Human norovirus was believed to be completely inactivated at 73°C for 2 min in a novel binding study correlating infectivity with capsid changes [5]. This last study by Dancho *et al.*, reports the correlation of receptors with infectivity and viability; a growing trend leading to the further development of binding and ELISA assays [15].

Environmental persistence

As virus contamination issues become more complicated, environmental persistence is increasingly important. MNV and TV showed similar persistence to chlorine concentrations in water at 0.2, 20, 200, and 2000 ppm [14•] and on alfalfa seeds [40]. At 2 ppm chlorine in water, TV was significantly inactivated; however, MNV was not, leading to a discussion that MNV may be better suited as a surrogate to human norovirus which is believed to be resistant to normal levels of chlorine in drinking water