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ORIGINAL PAPER

Critical Review of Norovirus Surrogates in Food Safety Research: Rationale for Considering Volunteer Studies

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Abstract The inability to propagate human norovirus (NoV) or to clearly differentiate infectious from noninfectious virus particles has led to the use of surrogate viruses, like feline calicivirus (FCV) and murine norovirus-1 (MNV), which are propagatable in cell culture. The use of surrogates is predicated on the assumption that they generally mimic the viruses they represent; however, studies are proving this concept invalid. In direct comparisons between FCV and MNV, their susceptibility to temperatures, environmental and food processing conditions, and disinfectants are dramatically different. Differences have also been noted between the inactivation of NoV and its surrogates, thus questioning the validity of surrogates. Considerable research funding is provided globally each year to conduct surrogate studies on NoVs; however, there is little demonstrated benefit derived from these studies in regard to the development of virus inactivation techniques or food processing strategies. Human challenge studies are needed to determine which processing techniques are effective in reducing NoVs in foods. A major obstacle to clinical trials on NoVs is the perception that such trials are too costly and risky, but in reality, there is far more cost and risk in allowing millions of unsuspecting consumers to contract NoV illness each year, when practical interventions are only a few volunteer studies away. A number of clinical trials have been conducted, providing important insights into NoV inactivation. A shift in research priorities from surrogate research to volunteer studies is essential if we are to identify realistic, practical, and scientifically valid processing approaches to improve food safety.

Keywords Norovirus inactivation · Surrogate · Feline calicivirus · Murine norovirus · Clinical trials · Food safety · Volunteer study

Introduction

Human noroviruses (NoVs) are a primary cause of viral gastroenteritis throughout the world (Siebenga et al. 2009), and the principle cause of foodborne illness in Europe (Kroneman et al. 2008; Phillips et al. 2010) and the United States (Mead et al. 1999; Scallan et al. 2011). The number of estimated cases in the United States was recently revised to 5.5 million annually (Scallan et al. 2011), while England has an estimated 2 million cases per year (Phillips et al. 2010). As enteric viruses, the NoVs are spread via the feces or vomitus of infected individuals. Norovirus illness is contracted through contaminated food and water and direct person-to-person transmission. Contamination often arises during the handling and preparation of foods, although there are many instances where foods, particularly shellfish, are contaminated within their environment. Products requiring extensive handling are also prone to contamination. In spite of interventions to eliminate product contamination, like thorough cooking, some products, such as salads and molluscan shellfish are typically eaten raw or

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only lightly cooked and such products represent the greatest risk to the consumer (Richards 2001; Richards et al. 2010).

The development of commercial processing strategies for foods has been largely based on reductions in bacterial pathogens, and only to a lesser extent on the inactivation of enteric viruses. For some of the enteric viruses, like poliovirus and related *Picornaviridae*, astroviruses, parvoviruses, rotaviruses, and adenoviruses 40 and 41, the levels of infectious virus particles can be determined using cell or tissue culture, since these viruses are propagatable. Unfortunately, NoV cannot be routinely propagated in cell culture or animal models. Human exposure and resulting illness are currently the only means to distinguish infectious from inactivated NoVs. Scientists and regulators have relied on virus detection in foods based on the physical presence of viral RNA, as determined by reverse transcription-PCR, which presently cannot distinguish between infectious and inactivated viruses (Richards 1999), although methods are being developed toward that goal (Li et al. 2011; Nuanualsuwan and Cliver 2002; Parshionikar et al. 2010). The lack of a suitable assay for infectious NoV and other unculturable viruses, like most wild-type strains of hepatitis A virus (HAV), has led the research community to focus on viral surrogates. Surrogates are viruses related to the pathogens they have been chosen to represent. The selection of a surrogate, at least in regard to NoVs, has been based on the ability of the surrogate to be propagated in culture, and its genetic, physical, or chemical relatedness to the pathogen. Over the years, different surrogates have been selected for use in determining the uptake, persistence, distribution, and inactivation of viruses in foods, water, and environmental samples as well as in chemical disinfection studies on surfaces. Studies with surrogates have been used with the intent to develop a better understanding about the pathogens they represent as well as applied applications for pathogen elimination.

A respiratory virus known as feline calicivirus (FCV) in the genus *Vesivirus* was first used as a NoV surrogate in the late 1990s (Doultree et al. 1999; Slomka and Appleton 1998). It has been commonly employed in studies involving chemical disinfectants (Cannon et al. 2006; D'Souza and Su 2010; Doultree et al. 1999; Gehrke et al. 2004; Hudson et al. 2007; Jimemez and Chiang 2006; Kampf et al. 2005; Lages et al. 2008; Malik et al. 2006; Malik and Goyal 2006; Mori et al. 2007; Morino et al. 2009; Poschetto et al. 2007; Sattar et al. 2011; Steinmann 2004; Urakami et al. 2007; Whitehead and McCue 2010); and processing interventions, like heating (Buckow et al. 2008; Butot et al. 2009; Cannon et al. 2006), freezing or freeze-drying (Butot et al. 2008, 2009), irradiation (de Roda Husman et al. 2004; Fino and Kniel 2008; Nuanualsuwan et al. 2002), marinating/acidification (Cannon et al. 2006; Hewitt and Greening 2004), and high pressure processing (Chen et al. 2005; Kingsley et al. 2002). A decade after FCV first came on the scene, Wobus et al. (2006) identified murine norovirus-1 (MNV) as a closer genetic relative of NoV. Now, MNV has become the more commonly used surrogate for chemical disinfection studies (Belliot et al. 2008; Cannon et al. 2006; D'Souza and Su 2010; Lee et al. 2008; Lim et al. 2010; Magulski et al. 2009; Park et al. 2010; Sattar et al. 2011); and studies on processing interventions like heating (Baert et al. 2008a; Cannon et al. 2006; Hewitt et al. 2009; Sow et al. 2011; Tanner 2009), freezing (Baert et al. 2008b), UV irradiation (Jean et al. 2011; Park et al. 2011), gamma irradiation (Feng et al. 2011), electron beam (Sanglay et al. 2011), and high pressure processing (Kingsley et al. 2007; Lou et al. 2011; Tang et al. 2010). Poliovirus and bacteriophage MS2 have also been used as potential surrogates for NoV (Bae and Schwab 2008; D'Souza and Su 2010; Dawson et al. 2005; Shin and Sobsey 2003). Another potential surrogate is the recently discovered rhesus monkey calicivirus, known as Tulane virus, which can replicate in cell culture, but as indicated by Tan and Jiang (2010), may not serve as a good model for human NoV because it is not in the norovirus genus and has not been shown to cause gastroenteritis like the human NoVs. Unfortunately, there are fundamental differences in the inactivation rates of many closely related viruses—differences which may limit their role as surrogates.

Limitations of Viral Surrogates

Surrogate viruses are generally expected to mimic the viruses they represent, although studies are proving this concept invalid. In direct comparisons between FCV and MNV, their susceptibility to temperatures, pH, and environmental conditions has been shown to be dramatically different (Cannon et al. 2006). In these studies, MNV was significantly more resistant to both acidic and alkaline pHs than FCV; MNV was more resistant than FCV to chloroform, Freon, and Vertrel; FCV was more stable than MNV at 56°C ($P < 0.05$), but differences were not significant at 63 and 72°C; and MNV was more stable in solution at room temperature (Cannon et al. 2006). Gibson and Schwab (2011) reported that FCV was significantly less stable than MNV at 50°C, but not significantly different at 60°C. In these same studies, HAV was significantly more resistant than FCV and MNV to heat treatment at 50 and 60°C (Gibson and Schwab 2011). Bae and Schwab (2008) evaluated the persistence of FCV, MNV, MS2 and poliovirus in surface water and groundwater and found that FCV was significantly less stable than MNV, MS2, and poliovirus. Sattar et al. (2011) compared the effects of

ethanol-based hand rubs to eliminate FCV and MNV. They showed that over a short contact time (20 s) that FCV was 100 times more resistant to inactivation than MNV. In another study on hand sanitizers, FCV was more sensitive to some low pH sanitizers than MNV; however, MNV was more readily inactivated by alcohols, thus the recommendation was made to include both surrogates when testing hand sanitizers (Park et al. 2010). A study on the inactivation of FCV and MNV by UV irradiation (254 nm) showed FCV to be more sensitive than MNV (Park et al. 2011). D'Souza and Su (2010) evaluated the effects of chemical treatments to inactivate FCV, MNV, and MS2 and reported that a 2% trisodium phosphate treatment for 1 min decreased FCV and MS2 by over 6 logs, but MNV by only 1 log. They also showed that 2% glutaraldehyde reduced FCV and MNV titers by 6 logs, but MS2 by <3 logs. Seventy percent ethanol was reported to cause no reduction in titers for any of the viruses (D'Souza and Su 2010). Ueki et al. (2007) compared the persistence of NoV and FCV in the digestive tissues of the oyster after depuration for 10 days and concluded that FCV was rapidly depleted, whereas NoV persisted. We now know that NoV binds to histo-blood group antigens (HBGAs) present on gastrointestinal cells of oysters, clams, and mussels (Le Guyader et al. 2006; Tian et al. 2006, 2007), which may account for differences in virus uptake by shellfish. Tulane virus also binds to HBGAs (Farkas et al. 2010), while MNV and FCV bind to sialic acid on the host cell surface (Stuart and Brown 2007; Taube et al. 2009). These results should lead one to conclude that not all virus surrogates are equal; some surrogates are more similar while others are quite different from the pathogens they represent.

A major limitation in these studies is a lack of correlation in the inactivation rates of the surrogates and of the pathogen. Koopmans and Duzier (2004) suggested the use of the most resistant enteric virus in developing food safety guidelines. This may over regulate the industry if NoV is substantially more susceptible than the surrogate. On the other hand, even the most resilient surrogate may not be as resistant as the pathogen, and may lead to a false sense of security relative to the safety of food or water. Unfortunately, there is often no information available to directly correlate the pathogen with its ascribed surrogate, leaving regulators unable to promulgate new food regulations based on surrogate studies. In addition, a surrogate may be representative of a pathogen's response to a particular processing scenario (e.g., pasteurization), but may not necessarily represent a pathogen's response under other processing conditions or in other food matrices. Data obtained must be carefully scrutinized and treated as presumptive evidence of how the pathogen may respond to a particular treatment. The use of FCV as a surrogate has diminished as more researchers are utilizing MNV, which is genetically more similar to NoV than FCV. Consequently, past FCV research is being looked on as unreliable, while MNV is rapidly gaining popularity as a more suitable surrogate, in spite of the fact that FCV seems more resistant to chemical disinfectants than MNV.

The question today is whether MNV is an adequate surrogate for NoV—one that closely mimics the virus it represents. Is MNV the answer? Probably not. The reason is that MNV may fail to respond in the same manner as the pathogen it represents. This was demonstrated in a volunteer study involving the inactivation of NoV in oysters using high pressure processing (Leon et al. 2011). In this study, pressures of up to 600 megaPascals (MPa) for 5 min at room temperature were required to totally eliminate 4 logs of NoV (Leon et al. 2011). In comparison, only 250 MPa was required to inactivate 7 logs of FCV in cell culture media under the same conditions, while 4 logs of MNV required 400 MPa for 5 min at 5°C for inactivation (Kingsley et al. 2002, 2007; Leon et al. 2011). One of the problems in comparing such studies is differences in processing conditions or matrix between assays. Here, we see different matrices (cell culture media and oysters) and different processing conditions (room temperature and 5°C); however, no other comparative studies exist for this processing technology.

Another example of the differences in the inactivation of related viruses may be seen for poliovirus and HAV. Poliovirus has been used as a surrogate for HAV because of its similarity in size, shape, and structure to HAV; however, high pressure processing can inactivate HAV at pressures around 400 MPa, but poliovirus persists at 600 MPa (Kingsley et al. 2002; Wilkinson et al. 2001). Another study showed that different strains of cell culture-adapted HAV have different sensitivities to heat and high pressure, where heating to 60°C for 10 h and pressures of 420 MPa reduced virus infectivity by anywhere from 3 to 5 logs, depending on the strain (Shimasaki et al. 2009). This represents a 100-fold difference in the inactivation of different strains of HAV and highlights how different strains of the same virus do not accurately portray the inactivation dynamics of all HAV strains. Much like the differences in inactivation noted among HAV strains, different responses to inactivation should be anticipated among the NoV strains. Different strains of FCV also showed widely differing susceptibilities to inactivation by three alcohol mixtures and a chlorine compound (Di Martino et al. 2010) and to pH and heat (Lee and Gillespie 1973). If strains of the same virus give discordant inactivation results, then one might expect virus surrogates, which are only slightly related to the pathogens, to be poor indicators of the pathogen's inactivation kinetics. NoVs include a variety of genetically similar strains (also called genotypes or clusters) within two genogroups (I and II). The uptake of

different NoV strains by oysters varied with genogroup I, cluster 1 NoV (Norwalk virus strain) efficiently bioconcentrated, but genogroup II, cluster 4 NoV poorly bioconcentrated (Maalouf et al. 2011). Consequently, the use of a surrogate for viruses that differ widely in their genetic composition and likely in their response to various processing techniques, chemical disinfection, or environmental conditions seems counterproductive. Although the most resistant surrogates may be used to evaluate processing effectiveness, the pathogens could be several orders of magnitude more resistant to treatment than the surrogate. Predictive models have been proposed for NoV and HAV inactivation in shellfish, based on the use of surrogates, either FCV or a cell culture-adapted strain of HAV (Buckow et al. 2008; Grove et al. 2009); however, such models are not likely to accurately portray the inactivation of pathogenic viruses on or within foods. With the likelihood that MNV will not accurately reflect the conditions necessary to inactivate NoV in foods, one questions the funding of surrogate studies. Will MNV become another FCV, with millions of dollars spent on research but little confidence in the results?

Costs and Benefits

Considerable research funding is provided globally each year to conduct surrogate studies on NoV; however, there is little benefit derived from these studies in regard to the development of food processing strategies. After years of research with FCV as the surrogate, investigators are considering those results highly presumptuous, particularly in light of MNV which is now generally perceived as a better surrogate. The time, effort, and cost that were devoted to surrogate research with FCV may have been misdirected. At what cost was the FCV research conducted? No one can be sure, but certainly millions of dollars have been spent on what is now considered by most as an ineffectual surrogate. A similar fate will befall MNV when researchers identify still better surrogates for NoV and similar or greater dollar losses can be expected. Uncertainty concerning the reliability of surrogate studies has, to the best of this author's knowledge, prevented results from being implemented in regulatory actions or new food processing procedures. If information were available on how much money was spent on surrogate studies and one were to conduct a cost–benefit analysis on the FCV or MNV studies performed to date, results would be startling, since there has been much money spent, but with little change in the way we process foods or sanitize the workplace. At the time of this writing (September, 2011), a number of papers had already been published during 2011 on the use of MNV as a NoV surrogate, including the use of varying processing technologies, like electron beam (Sanglay et al. 2011); gamma irradiation (Feng et al. 2011), and high pressure processing (Lou et al. 2011) to inactivate MNV in produce; heat inactivation of MNV in clams (Sow et al. 2011); chemical disinfectants to eliminate MNV on produce and food contact surfaces (Predmore and Li 2011) and hands (Park et al. 2011; Sattar et al. 2011); and pulsed UV light inactivation of MNV on food contact surfaces (Jean et al. 2011). Such studies fail to provide reasonable expectations that the surrogates respond in a similar manner to NoV or to different genogroups or strains of NoV. In spite of the well intentioned and competent research that has been performed on NoV surrogates, results derived from surrogate studies have not answered the important questions regarding NoV inactivation in foods, water, or on food contact surfaces. We need to ask ourselves if FCV or MNV could ever be used in establishing food processing regulations when they only provide a glimmer of how the pathogen might respond to certain conditions. With all this uncertainty about surrogates, how does one derive definitive answers concerning what methods are needed to inactivate NoVs? The answer is human clinical trials.

Need for Clinical Trials

Current practices to evaluate surrogate viruses and to employ molecular assays should be limited, particularly for NoV inactivation studies. It is hereby recommended that presumptive information derived from the use of surrogates be subjected to proof-of-principle testing and validated in volunteer studies using NoV. Human challenge studies are essential to determine which processing techniques are effective in reducing NoV in foods. NoV is considered the primary cause of gastrointestinal illness worldwide. With such high morbidity rates, strategically designed volunteer studies performed under controlled conditions would seem prudent in order to assess the effects of cooking, freezing, irradiation, disinfectants, and other processing technologies on virus inactivation. The cost for challenge studies would be high; however, the results would be definitive. A case in point was the recent volunteer study to assess the effectiveness of high pressure processing to inactivate NoV in oysters, where it was definitively shown that pressure of up to 600 MPa for 5 min at room temperature would be required to inactivate 4 logs of the genogroup I.1 (Norwalk virus) strain of NoV (Leon et al. 2011). Does this mean that the same processing conditions would be required to inactivate the same level of other NoV strains? No, not at all, but is seems likely that differences between NoV strains would be less than differences between NoVs and their so called surrogates.

Another example of the benefit of clinical trials involved a study performed in Australia where shellfish containing NoV were "purified" by depuration (Grohmann et al. 1981). Depurated oysters were subsequently fed to volunteers who became ill, thus demonstrating that commercial depuration was not effective in eliminating NoV in contaminated oysters (Grohmann et al. 1981). In this case, volunteer studies provided definitive answers about the infectiousness of the shellfish. Volunteer studies can provide a firm basis for developing food and water processing strategies and for making regulatory decisions. Until such time that researchers develop a cell culture propagation system for NoV, or other means to discriminate infectious from inactive NoV, human challenge studies are our best hope for determining true virus inactivation parameters.

This recommendation to limit surrogate studies will be highly controversial, since funding for surrogate research has been plentiful and many researchers (including those in my laboratory) are accustomed to conducting NoV research with virus surrogates. However, a shift in research directions is essential if we are to identify realistic and practical processing strategies to improve food safety. Former and present research conducted with NoV surrogates will likely contribute little to improving the world's food supply, while the costs of such studies could be pooled and redirected to more definitive clinical trials on NoV inactivation. Future surrogate studies should be considered only when the research is well justified with a clear delineation of why a study with surrogates is appropriate and what definitive information is anticipated. Surrogate studies should be linked, to the extent possible, with NoV volunteer studies to determine if the inactivation rates of surrogates and NoVs correlate after various processing interventions. Similar inactivation profiles would validate the surrogate for a particular use under defined conditions.

Clinical trials will again be necessary to validate some of the new and upcoming assays that are designed to differentiate infectious from inactivated viruses, such as pretreatment of viruses with proteinase K and ribonuclease before RT-PCR (Nuanualsuwan and Cliver 2002), use of ethidium and propidium monoazide in conjunction with RT-PCR assays (Kim et al. 2011; Parshionikar et al. 2010), and various receptor and binding assays, like one developed by Li et al. (2011) using MNV. Virus surrogates may play a role in the development of assays for NoV infectivity; however, confirmation that the surrogates are truly representative of NoV must be accomplished by volunteer studies. For instance, receptor binding assays which show that MNV only binds when it is infectious, should be linked with volunteer studies designed to evaluate the assay using NoV of demonstrated infectivity, as determined by clinical trials. Many in vitro surrogate studies suggest that NoV can be inactivated by one treatment or another, but fail to carry the work forward to validate the results using similar treatments of NoV in volunteer studies.

Over the years, a number of NoV volunteer studies have been performed. My laboratory just completed a 5-year collaborative study that involved clinical trials at Emory University in Atlanta (Leon et al. 2011). Since 1994, there were seven separate trials involving 190 volunteers at Emory University and the University of North Carolina at Chapel Hill (Lindesmith et al. 2003, 2005, 2010) and there were no adverse reactions among any of the volunteers (personal communication, Christine Moe, Emory University). Other clinical trials involving NoVs have been performed at Baylor College of Medicine (Graham et al. 1994; Hutson et al. 2002, 2005) and elsewhere (Wyatt et al. 1974; Dolin et al. 1982). NoV illness is usually a temporary imposition where adverse effects outside of the usual nausea, diarrhea, and vomiting are seldom encountered; therefore, there is reasonable expectation that trials may be conducted with minimal risk of complications. Oversight of clinical trials must be provided by institutional review boards for human subjects or similar entities whose mission is to evaluate potential patient risks, to ensure they are minimized to the extent possible, and to weigh the risks versus benefits of the proposed research. Costs for studies vary greatly, but an average volunteer may expect compensation of approximately $1000 (US) for their participation and incarceration in the hospital for 3 or 4 days. Hospital costs, labor, and overhead for researchers add to the overall expense, thus the cost of a volunteer study may approach $500,000 (US) in today's market, depending on the number of volunteers required to provide statistically significant results. If monies that were normally used for surrogate studies were pooled, then multiple volunteer studies could likely be funded each year.

One obstacle to clinical trials is the perception that such trials are risky. The threat of lawsuits, in the event of unforeseen circumstances, prevents some governments, universities, and hospitals from conducting or considering the funding of such studies. In reality, there is far more risk in allowing millions of unsuspecting consumers to contract NoV each year, when practical interventions are only a few volunteer studies away. The price to pay for inactivity is great, with lost wages, medical costs, and regulatory and epidemiological expenses involved in tracking and managing outbreaks. Costs of supporting clinical trials would be a small price to pay for the considerable savings that would be brought about by even a modest reduction in NoV outbreaks. A change in attitudes and a shift in research priorities are essential if we are to win the battle against NoV illness.

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