Comprehensive Comparison of Cultivable Norovirus Surrogates in Response to Different Inactivation and Disinfection Treatments

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Running title: Comparison of inactivation and disinfection patterns of surrogate viruses for human norovirus

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ABSTRACT

Human norovirus is the leading cause of epidemic and sporadic acute gastroenteritis. Since no cell culture method for human norovirus exists, cultivable surrogate viruses (CSV) including feline calicivirus (FCV), murine norovirus (MNV), porcine enteric calicivirus (PEC) and Tulane virus (TuV) have been used to study the response to inactivation and disinfection methods. We compared the infectivity reduction of CSV and Aichi virus (AiV) by extreme pH, 56°C heating, alcohols, chlorine on surfaces, and high hydrostatic pressure (HHP) using the same matrix and identical test parameters for all viruses, and compared with reduction of human norovirus RNA. At pH 2 FCV was inactivated by 6 log_{10} whereas MNV, TuV and AiV were resistant. All CSV were completely inactivated at 56°C within 20 min. MNV was inactivated 5 log_{10} by alcohols compared to 2 and 3 log_{10} for FCV and PEC, respectively. TuV and AiV were relatively insensitive to alcohols. FCV was reduced 5 log_{10} by 1,000 ppm chlorine compared to 1 log_{10} for the other CSV. All CSV except FCV dried on stainless steel surfaces were insensitive to 200 ppm chlorine. HHP completely inactivated FCV, MNV, and PEC at ≥300 megapascal (MPa), TuV at 600 MPa while AiV was completely resistant up to 800 MPa. By RT-qPCR, genogroup (G)I norovirus was more sensitive than GII norovirus to alcohols, chlorine, and HHP. Although inactivation profiles were variable for each treatment, overall TuV and MNV were the most resistant CSV and therefore are best candidates to study public health outcomes of norovirus infections.
INTRODUCTION

Noroviruses are the leading cause of epidemic and sporadic acute gastroenteritis (1, 2) and infect people of all ages worldwide. The primary mode of transmission is person-to-person (3, 4), but foodborne transmission also plays a significant role in outbreaks (5). Transmission of norovirus disease is promoted by several factors including a large human reservoir, shedding of large amounts of virus ($10^{5-9}$ particles/g stool), prolonged virus shedding, environmental stability, relative resistance to disinfection, and environmental contamination (6). Over the past decade, the development of sensitive molecular techniques has provided a better understanding of the burden and molecular epidemiology of norovirus disease and has been useful in the detection of norovirus in a variety of environmental settings. Despite significant efforts, human noroviruses cannot be cultivated in cell culture (7-10) and therefore the effect of physiochemical exposure, specific disinfectants, or how to best inactivate infectious viruses cannot be evaluated quantitatively. Consequently, the most common approach to understand the response to disinfectants and the environmental stability of infectious norovirus has been the use of cultivable surrogate viruses, while detection of viral RNA by RT-qPCR has been employed to compare the effects of inactivation methods on viral RNA (11). The latter approach, however, does not provide information about virus infectivity, and only a small region of the RNA genome is amplified which may underestimate the efficacy of treatments.

Surrogates have most commonly been used in microbiology as indicators within an environmental context such as water or food to represent potential presence of pathogens (12). Surrogates should be considered to be used when no methods are available to study the effects of physiochemical conditions or inactivation methods for a pathogen. An ideal
surrogate should have similar biological, biochemical and biophysical characteristics as the pathogen of interest. Noroviruses are non-enveloped, single stranded RNA viruses 27 to 40 nm in diameter. They belong to the family *Caliciviridae* which consists of five genera: Norovirus, Sapovirus, Lagovirus, Vesivirus, and Nebovirus, and three proposed genera, Recovirus, Valovirus, and chicken calicivirus (13). In 2008, Tulane virus (TuV) was first described as the first member of the proposed genus Recovirus (14). Viruses from the calicivirus family are the logical surrogate choice, and because feline calicivirus (FCV) is cultivable, this virus has been used as the preferred surrogate for norovirus in numerous studies since the 1970’s (15). With the discovery of murine norovirus (MNV) in 2003 (16), many researchers are now using this newly described virus as it is genetically more closely related to human noroviruses, and resistant to low pH (17, 18). Many studies comparing FCV and MNV with or without other surrogates such as MS2 coliphage, hepatitis A virus, as well as human norovirus (using RT-qPCR) have been published (4, 18-24). However, as MNV is highly sensitive to alcohols (18), the focus has expanded to cultivable caliciviruses such as TuV (14, 25-28), and porcine enteric calicivirus (PEC), a GIII sapovirus (29, 30).

Although numerous studies have been published comparing two or more of the potential norovirus surrogate viruses, none have simultaneously compared all the cultivable caliciviruses. Furthermore, data across studies is difficult to compare because inactivation conditions such as exposure time, concentrations of disinfectants and the final matrix have not been consistent. In this study, we compared four surrogates of human norovirus head to head using identical conditions. In addition, Aichi virus (AiV) was also included because of its established environmental stability (31). AiV was first detected in humans in 1989 (32) and has...
since been detected in water and sewage worldwide (33). We chose to compare five virus
inactivation methods including physiochemical treatments (heat and pH), commonly used
disinfectants (chlorine and alcohol), and high hydrostatic pressure (HHP) as an emerging
method for the inactivation of pathogens in food (34). Inactivation was measured by
quantitative loss of infectivity for each of the five viruses. In addition, selected conditions and
surrogate viruses were compared with human norovirus for their effect on viral RNA.
MATERIALS AND METHODS

Viruses, cells, and infectivity assays  
FCV strain F9 (ATCC VR-782, Rockville, MD) was propagated in CRFK cells (ATCC CCL-94) in EMEM (MEM with Earle’s salts, Gibco Life Technologies, Grand Island, NY) plus 10% fetal bovine serum (FBS) (Hyclone, Logan, Utah) as previously described (22). Confluent monolayers of CRFK were inoculated with stock virus into the growth media and harvested at 2 days post infection (PI) when CPE was complete by freezing and thawing three times followed by centrifugation for 30 min at 3000 X g at 4°C. Aliquots of FCV (1 x 10⁹ PFU/ml) were stored at -70°C, and each aliquot was freshly thawed for each experiment and only used once. All virus aliquots, experimental controls and treated samples were assayed by plaque assay in 60 mm dishes as previously described (22).

MNV strain CW3 (a gift from Dr. Skip Virgin, Washington University, St. Louis, MO) was propagated in RAW 264.7 cells (ATCC TIB-71) in DMEM (Dulbecco’s MEM with no pyruvate, plus 10% low endotoxin FBS (Hyclone, Logan, UT). Infectious MNV was assayed by inoculation of 60 mm dishes of RAW monolayer cells with 500 µl of 10-fold serial dilutions, incubation for one hour at 37°C with rotation, followed by adding an overlay with medium consisting of MEM +2% FBS and 0.5% agarose. At 2 day PI, a second agarose overlay containing 66 mg/ml neutral red (Sigma, St. Louis, MO) was added and visible plaques were counted within 8 h. MNV frozen aliquots had titers of 10 ⁷-⁸ PFU/ml.

AiV, a gift from Dr. Pierre Poitier (Dijon University Hospital, Lyon, France), was propagated and assayed by plaque assay in Vero cells (ATCC CCl-81) using the same protocols as described for FCV above and had a titer of 1x 10⁷ PFU/ml.
TuV (14) was propagated in LLC-MK2 cells cultured in Opti-MEM (Gibco Life Technologies, Grand Island, NY) +2% FBS. Monolayer of cells were infected for 1 h at 37°C at 0.001 MOI in 10 ml of serum free media onto T150 flasks, followed by addition of 40 ml Opti-MEM + 2% FBS. Virus was harvested at 48-72 h when CPE was complete, clarified at 3,000 x g for 30 min, and frozen in aliquots at -70°C until use. Virus titers were determined by plaque assay which was complete as early as 48 h PI or could be enumerated after overnight incubation. TuV plaque assay was performed similar to FCV, AiV, and MNV except that viral dilutions were made in Opti-MEM + 2%FBS. As described for MNV, after 2 days an agarose overlay containing neutral red was used to stain plaques. The TuV titer was 10^7 PFU/ml.

Porcine enteric calicivirus (PEC/Cowden strain) was obtained from Dr. Linda Saif (The Ohio State University) (29). LLC-PK cells (ATCC CL-101) were passaged in MEM + 5% FBS every 3 to 5 days, as previously described (30). Briefly, three day old monolayers were rinsed two times with MEM prior to infection with an MOI of 0.01. After virus adsorption for 1 h, complete MEM + 2% FBS containing 100 mM GCDCA (glycochenodeoxycholic acid sodium salt, Sigma) was added to the LLC-LLC-PK cell monolayers. Complete CPE appeared at 72 h PI. Flasks were frozen and thawed three times, and cell debris were removed by centrifugation at 3,000 x g for 30 min to obtain serum free virus aliquots which were stored at -70°C. The PEC titer was 0.38 x 10^6 TCID_{50}/ml. Ninety-six well plates (CoStar, Corning Inc., Corning, NY) of LLC-PK cells were inoculated with PEC treated or untreated samples. Infectious PEC virus was measured by a previously described TCID_{50} assay (35) and virus was detected by using a porcine anti-PEC polyclonal serum (kindly provided by Dr. Linda Saif) followed by HRP-labeled goat anti-swine (KPL, Gaithersburg, MD), and color developed by AEC development kit (Sigma, St. Louis, MO).
Cells were observed by microscopy for reddish staining of cytoplasm to enumerate positives. The results of 5 wells of each inoculum were used to calculate the $TDI_{50}$ titer by the Reed and Muench Method (36).

Clinical samples, preparation and viral RNA extraction: GI.1 norovirus positive fecal specimens were kindly provided by Dr. Christine Moe, Emory University. GI.5 and GII.13 norovirus positive fecal specimens were obtained from outbreaks submitted to the National Calicivirus Laboratory at CDC. Clarified 10% fecal suspensions were prepared in PBS pH 7.2 and centrifuged at 10,000 x $g$, 30 min at 4°C. To prepare semi-purified (SP) stool preparations, the clarified stool suspensions were buffer exchanged into MEM + 10% FBS using an Amicon™ 50 K Ultra-15 ultrafilter (Millipore, Billerica, MA). Viral RNA concentrations were determined by norovirus GI/GII Taqman™ real-time RT-PCR as described below. The GI.1, GI.5 and GII.13 suspensions contained 4.69 x $10^5$ RNA copies/µl, 1.47 x $10^4$ RNA copies/µl and 5.94 x $10^6$ RNA copies/µl, respectively. The GI.5 SP contained 1.95 x $10^5$ RNA copies/µl and the GII.13 SP preparation contained 1.23 x $10^7$ RNA copies/µl. Norovirus preparations for experimental work were stored in aliquots at -70°C, and thawed aliquots were used only once.

RNase treatment prior to nucleic acid extraction was performed by incubation with RNaseOne™ Ribonuclease (1U/µl) (Promega, Madison, WI) at 37°C for 2 hours and stopped by the addition of SDS to a final concentration of 0.1%. Viral nucleic acid from control and treated samples was extracted using the KingFisher® instrument and MagMAX™ Total RNA Isolation Kit (Ambion, Austin, TX), according to the manufacturer's instructions.
Taqman™ real-time RT-PCR (RT-qPCR)  All RT-qPCR assays were TaqMan™-based and performed using the Ag Path One Step RT-PCR Kit (Ambion, Austin, TX) or QuantiTect Probe RT-PCR kit (Qiagen, Valencia, CA) on an Applied Biosystems 7500 platform (Applied Biosystems, Foster City, CA). GI/GII norovirus RNA was detected as previously described (37). Two standard curves consisting of 10-fold serial dilutions of GI.4 RNA transcripts and GII.7 RNA transcripts were included in each assay as previously described (38). MNV, FCV and AiV RT-qPCR were performed as previously described (22, 39).

TuV RT-qPCR was performed in a 25 µl reaction volume consisting of 3 µl of template RNA, 12.5 µl of 2X RT-PCR Buffer (Ag-Path One-Step RT-PCR Kit, Ambion, Austin, TX), 0.6 µl of each oligonucleotide primer (400 nM), 0.3 µl (200 mM) of TaqMan™ probe, 1 µl of 25X RT-PCR Mix (Ag-Path One-Step RT-PCR Kit, Ambion, Austin, TX), and RNase free-water. The amplification conditions consisted of an RT step of 10 min at 45°C, followed by 10 min at 95°C to activate the Taq polymerase, then 40 cycles of amplification for 15 s at 95°C and 1 min at 60°C. The oligonucleotide TaqMan™ probe (FAM-5’-TTTTCCATC[C]CATTCA[C]AAGT-3’-BHQ, with internal G-clamp noted as [C], and primers (TuV- F 5’-TTCACCCGACCAACCCTG-3’, and TuV-R 5’-ACGCCCAACGCACCTA-3’) were designed to target ORF-1 of TuV (GenBank accession number: EU391643.1) (14).

A standard curve for each virus was generated by amplifying 10-fold serial dilutions of FCV, MNV, TuV and AiV viruses. The Ct values for each dilution were used to plot a standard curve against the corresponding PFU/ml. Concentration for each sample (treated or control)
was extrapolated from this curve. GI and GII viral RNA copies/µl were calculated from transcript standard curves that were included in each experimental assay.

**pH treatment** Each virus aliquot (FCV, MNV, AiV, TuV) was diluted 1:10 into 100 mM citric acid buffer solutions at pH 2, 3 and into 100 mM carbonate buffer at pH 9 or 10 in 4.0 ml polystyrene tubes. After 30 min at 37°C, reactions were stopped by diluting 1:10 into DMEM or MEM +2%FBS to adjust pH to 7.0-8.0. Therefore the virus titers in the treated samples were 1:100 dilutions of stock viruses. Experimental controls were also incubated for 30 min at 37°C.

**Heat treatment** Virus aliquots were diluted 1:10 in 0.017 M PBS pH 7.4 (Hyclone, Logan, UT) and inactivated at 56°C in a digital dry bath (Labnet International, Inc.) for 2, 5, 10 or 20 min and the treatment was stopped by transferring the 1.8 ml microfuge tubes containing 1.0 ml of diluted sample to an ice bath.

**Alcohol treatment** Virus aliquots were diluted 1:10 into the test alcohol solutions for a final 1.0 ml volume and incubated for the indicated times 1.8 ml microfuge tubes, followed by dilution 1:10 into EMEM +10% FBS to stop inactivation. Alcohol solutions were made by addition of the appropriate volume of cell culture grade water (Life Technologies, Grand Island, NY) to molecular biology grade absolute ethanol or isopropanol (Fisher Scientific, Fairlawn, NJ).

**Chlorine surface disinfection** We used a modification of a previously described method for the assessment of decontamination of surfaces (40). Ten µl of each virus in EMEM, DMEM or Opti-MEM (each containing 10% FBS) was dried on 16 mm stainless steel discs (Muzeen and Blythe Ltd., Winnipeg, Manitoba, CA) which were placed inside 24 well plates in a laminar flow hood for 1-1.5 h. Fifty µl of chlorine solution at 200 ppm or 1,000 ppm, prepared by dilution of
commercial bleach (Clorox, 6% sodium hypochlorite) in distilled water, was added to each dried
virus for 5 min. Disinfection was stopped by addition of 450 µl of 0.1% thiosulfate in MEM+10%
FBS. The solution was removed after vigorous repipetting onto the disc 6 to 8 times. Duplicate
control discs with only MEM treatment were included to calculate the amount of virus
recovery.

**High hydrostatic pressure treatment (HHP)** All viruses that were not prepared in
EMEM+10% FBS were buffer exchanged to EMEM+10% FBS on Amicon™ Ultra-15 50K
centrifugal filters (Millipore™, Billerica, MA) prior to shipment on dry ice to the Institute for
Food Safety and Health, Illinois Institute Technology for HHP treatment. One ml of virus aliquot
was filled into the shaft of a Pasteur pipette, heat sealed (Clamco Heat Sealer Model 252B,
Cleveland, Ohio), and then vacuum sealed in 2-mil 8 X 10 inch polyethylene pouches (Prime
Source, St Louis, MO). The pouches were then placed in heat sealed high barrier bags
containing 5,000 ppm sodium hypochlorite and transported on ice to the HHP bay. An Avure
24L high-pressure unit (Avure Technologies, Middleton, OH) with processing water cooled to
4°C was used. Samples were treated at the indicated pressures measured in megapascal (MPa)
for 1 min at 4°C. Pressure come-up time of 55-65 s was not included and release was
instantaneous. HHP treated samples were transferred to 1.7 ml cryovial tubes, immediately
frozen, and shipped on dry-ice to CDC for plaque assay, TCID$_{50}$ or RT-qPCR. All experiments
were performed in duplicate for each shipment and two separate shipments were made for
each virus producing at least four replicates for each parameter. Virus control samples were
also shipped, thawed and refrozen and returned to CDC to be used as the experimental
controls. In some cases as many as 6 replicate data points for a parameter were obtained.
**Data analysis.** All experiments were performed in duplicate with at least two or more replicates for each experiment. Virucidal activity of the treatments was determined by calculating the infectivity or RNA reduction as the difference between \( \log_{10} \) of control (PFU/ml or RNA copies/µl) and treated samples. Four or more log reduction values were averaged and the standard deviation determined. RT-qPCR data are the mean of 4 or more replicate samples and triplicate assay of each sample by RT-qPCR. Statistical comparisons were made with the Tukey HSD analysis using PASW Statistic 18 (IBM SPSS Inc, New York, NY) (41).

**RESULTS**

**pH stability**  We exposed the surrogate viruses to pH 2 and 3 as well as pH 9 and 10 for 30 min at 37°C (Figure 1). Of all viruses, FCV was the most susceptible to pH extremes of 2, 3 and 10 (p<0.0001). PEC was reduced by 1 log\(_{10}\) at pH 2, whereas MNV and TuV were reduced by less than 0.5 log\(_{10}\) each.

**Heat treatment**  Heat treatment is commonly used to destroy viruses within food and sometimes used to treat contaminated items. A common treatment is pasteurization of milk at 72°C for 15 s. Inactivation at a lower temperature for longer time periods can differentiate the kinetic differences between viruses. All viruses were inactivated rapidly at 60°C and 63°C (data not shown). Fifty-six degrees was chosen as a good temperature to observe kinetics of inactivation in a reasonable time. Up to 20 min of exposure at 56°C, increasing reduction of infectivity of each of the viruses was found (Figure 2). Maximum measurable virus reduction of 5 log\(_{10}\) was obtained at 20 min for FCV, MNV, and TuV. Near complete (four of 5 measurable}
log$_{10}$ reduction of AiV infectivity was achieved after 20 min of treatment. The virus reduction of PEC was less than that of the other viruses, 2 log$_{10}$ vs 4 to 5 log$_{10}$, which may be partial explained by the lower initial virus titer (0.38 x 10$^6$ TCID50/ml). Greater variation was found among replicate experiments for heat inactivation than for other test conditions.

**Alcohol sensitivity** Each surrogate virus, in suspension, was exposed to four different alcohol solutions (70% and 90% isopropanol; 70% and 90% ethanol) for 1 min and 5 min (Figure 3). MNV and PEC were the most sensitive to all test conditions with complete reduction (6 log$_{10}$) of MNV infectivity after 5 min exposure to 90% ethanol. PEC infectivity was reduced completely by all treatments. Each virus reduction level was similar after exposure to 70% alcohols at 1 min or 5 min, only MNV exposure to isopropanol showed a difference between exposure at 1 and 5 min. Exposure to 90% alcohols produced similar reductions, except MNV which showed greater reduction when exposed to 90% than 70% isopropanol. Treatment with 70% ethanol reduced FCV infectivity more than treatment with 90% ethanol at 1 min and 5 min, as previously observed (22). AiV and TuV lost less than 0.5 log$_{10}$ of virus titer with any of the treatments.

Because MNV infectivity was the most sensitive to alcohol treatments, we compared reduction of MNV RNA with RNA of GI.1, GI.5, GI.5SP, GII.13 and GII.13SP after 1 min of exposure to 70% or 90% ethanol solutions (Figure 4). MNV infectivity reduction was in the range of that seen in all previous experiments (Figure 3) and is near complete reduction of the measurable infectious virus. MNV RNA was reduced by 4-5 log$_{10}$ in the same samples. GI.5 SP RNA was reduced by as much as 3.5 log$_{10}$, whereas GI.13 and GI.13 SP were reduced by less
than 1 log_{10}. Reduction of G1.1, of G1.5, and of GII.13 RNA, after exposure to 70% or 90%
ethanol for 1 min, differed significantly from each other (p<0.05). There was a significant
difference between the reduction of the G1.5 SP and GII.13 SP compared with the non-semi-
purified preparations (G1.5 and GII.13) (p<0.05).

**Chlorine treatment on discs** After drying on stainless steel discs, viruses were exposed to 200
or 1,000 ppm chlorine for 5 min and evaluated for infectivity (Table 1). Less than one log_{10}
reduction was seen for any of the viruses after exposure to 200 ppm. FCV was reduced by 5
log_{10} after exposure to 1,000 ppm (p<0.0001), whereas the titers of the other viruses were
reduced by about 1 log_{10} (p<0.0001).

Nucleic acid extracts of the same samples were tested by RT-qPCR (Fig 5). After
exposure to 1,000 ppm chlorine, FCV RNA was reduced by 2.5 log_{10}. In contrast, TuV, MNV, and
AiV RNA levels were reduced by less than 0.5 log_{10} following treatment with 1,000 ppm
chlorine. G1.5 SP RNA was reduced by <1 log_{10} after 200 and 1,000 ppm chlorine treatment.
GII.13 SP RNA was reduced by less than 0.5 log_{10}.

**HHP treatment** All CSV and AiV were tested for loss of infectivity when treated with increasing
MPa (100-800) for 1 min at 4°C (Figure 6). AiV infectivity was not reduced by pressure as high
as 800 MPa whereas FCV infectivity was reduced by 6 log_{10} at 300 MPa, and MNV required 400
MPa for reduction of 6 log_{10}. PEC was completely inactivated (5 log_{10}) at 400 MPa. Although
TuV infectivity was reduced by 1 log_{10} at 100 MPa, in contrast to no reduction of the other
viruses at 100 MPa, 600 MPa was required for complete inactivation of TuV. The inactivation
kinetics appeared quite different from the other caliciviruses.
In another series of experiments reduction of RNA from human norovirus positive semi-purified stool samples (GI.5 SP and GII.13 SP) was compared with TuV RNA (Fig 7). At 200 MPa TuV RNA was reduced 1 log$_{10}$ with no further reduction of RNA up to 800 MPa. This was in contrast to the increasing reduction of TuV infectivity which reached its maximum reduction level at 600 MPa. GI.5 SP RNA was reduced 2 log$_{10}$ with 300 MPa treatment and no further reduction was seen at higher pressures, whereas, GII.13 SP RNA was reduced 1 log$_{10}$ at 400 MPa with no further reduction with increasing MPa.
DISCUSSION

In the absence of a cell culture system for human noroviruses, cultivable surrogate viruses have been used for decades even before the current understanding of the classification and characteristics of noroviruses. We compared the inactivation profiles of four cultivable viruses of the family *Caliciviridae* and *AiV*, which belongs to the family *Picornaviridae*, as surrogate viruses for the non-cultivable human noroviruses using identical experimental conditions. Although *AiV* is the most resistant virus to all treatments, a calicivirus surrogate would be the best indicator to assess the efficacy of inactivation methods against human norovirus. All four calicivirus surrogates demonstrated different inactivation patterns. However, we confirmed results from previous studies (18, 42) that FCV is much more sensitive than the other caliciviruses to low pH at which enteric viruses, such as human norovirus, need to survive to initiate infection. *PEC*, a sapovirus, is also less resistant to lower pH than the other surrogate viruses. In addition, in comparison to the other viruses, routine laboratory testing for *PEC* is a challenge because of its, compared to other surrogate viruses, low virus titer and the need for additional incubation steps with *PEC*-specific antibodies for detection. Because of the importance of low pH resistance and the need for relatively simple methods to use surrogates routinely, FCV and *PEC* are inadequate to assess the efficacy of methods to reduce the public health risk of norovirus infections. Based on our data and that of others, *MNV* and *TuV* are the most promising candidates to serve as surrogates for human norovirus (26). However, *MNV* is significantly more susceptible to alcohols in comparison with other non-enveloped viruses, as numerous studies have shown (19, 20, 22, 43). Alcohols, at a concentration of 60-95% (44), are important components of many hand sanitizers and commonly used in settings where bacteria
and viruses are pathogens of concern. We demonstrated that the level of reduction of MNV and TuV infectivity by chlorine was very similar, as was their sensitivity to low pH. The kinetics of reduction of infectivity at 56°C was slightly different, but both viruses lost almost all their infectivity after 20 minutes. The inactivation pattern of TuV with HHP treatment was notably different compared to other viruses and required 600 MPa to eliminate all infectivity, in contrast to 300-400 MPa required for inactivation of the other norovirus surrogates. HHP is an important parameter because of growing interest for its use in food preparation (45-49). We have demonstrated that across different inactivation procedures, TuV is the most resistant of the norovirus surrogates. In addition, TuV consist of at least 4 different genotypes which display diverse HBGA binding patterns mimicking human noroviruses (50).

As other investigators have shown, we found that RNA quantitation after treatment of surrogate viruses with chlorine, alcohols, or HHP, does not quantitate the loss of virus infectivity (22, 23, 47, 51). Although in some treatments an increase in RNA reduction did parallel an increase in infectivity reduction, as was apparent for the chlorine treatments of FCV and alcohol treatments of MNV. Interestingly, treatments of human norovirus with chlorine, alcohols, and HHP demonstrated that GII viruses are more resistant than GI viruses as measured by reduction of RNA by RT-qPCR. In contrast to our ethanol tolerance data for a GI.1 and a GII.13 virus, which were in agreement with published work (20, 22), we found 3log_{10} reduction for a semi-purified GI.5 virus. This suggests that differences in alcohol tolerance between different norovirus strains may exist and that purifying virus from stools need to be considered when testing more strains of both genogroups. Such results are of particular interest as alcohols are a key component of many hand sanitizers (22, 52, 53).
Overall, our results for the individual viruses are in general agreement with the results reported in previous studies (18, 20, 26, 27, 30, 42). However, it is difficult to make a direct comparison due to the great variety of certain test parameters (e.g., exposure time) between methods. In several instances where a one or two log reduction difference was found between our results and those of others, the methods employed did not include the exact same test conditions. PEC titers were reduced 1-2 log_{10} at extreme pH values, which were not evaluated in a previous study (30). Both TuV and MNV were stable at lower pH values and showed similar sensitivity at pH 10. Other investigators found TuV to be more sensitive to pH 2 and 10 than we found, possibly due to differences in experimental parameters (26). FCV and MNV were equally sensitive to heating at 56°C in our experiments with inactivation complete by 20 minutes, in agreement with a previous study (18). Our 56°C inactivation data for PEC are in the same range to what has been reported previously for these viruses (27, 30) whereas Tian et al. reported 3.5 log_{10} reduction of TuV compared to 5 log_{10} after 10 minutes of exposure that we found (25).

Our data confirms that MNV is more sensitive to alcohols than FCV (22, 30, 54) and that FCV is more sensitive to 70% ethanol than to 90% ethanol (22). The data for PEC is in line with previously published data indicating greater than 2 log reduction at 30 s (30). However, in contrast to what has been reported previously (24), we found that TuV infectivity was very marginally reduced by ethanol and isopropanol, similar to AiV. To compare the sensitivity of different surrogate viruses to chlorine, we chose to first dry the viruses on stainless steel coupons rather than measuring disinfection in solution to better mimic the environment of a food preparation area. Also, a simple dilution of commercial bleach in water was used without pH adjustment, as would be done in a food preparation environment (30, 54). In wastewater,
FCV is more susceptible to chlorine than polioviruses (55). In contrast to data on sensitivity of FCV to chlorine in solution (20), we found <0.5 log₁₀ reduction of infectivity or decline of FCV RNA when exposed to 200 ppm of chlorine which is similar to published data of FCV dried on a surface (24, 56). At higher chlorine concentrations (1,000 ppm), FCV was the only surrogate that was inactivated by more than 5 log₁₀, whereas the infectivity of the other viruses including TuV was reduced by about one log₁₀. This finding is in contrast with data from other studies that reported several logs of virus inactivation for MNV (26, 57) and TuV (26, 27) in solution. However, our data is in agreement with another study that also evaluated chlorine disinfection on stainless steel surfaces and found that FCV was more sensitive than MNV (23). Taken together, it is clear that these viruses are more difficult to inactivate on surfaces than in solution, which was previously demonstrated for MS2, MNV and human norovirus (58). Therefore, 200 ppm of chlorine (59) may not be sufficient to inactivate human norovirus on food contact surfaces.

HHP is an attractive option for inactivation of potential pathogens in food when raw character, flavor, and texture are important, such as shellfish, fruits (e.g., raspberries) and vegetable products. HHP is currently used to inactivate *Vibrio vulnificus* in shellfish (60), and a pressure of 275-300 MPa for several minutes is used in oysters (61). Inactivation of surrogates in other foods such as milk, juice, strawberry puree and blueberries will expand the application of HHP pathogen inactivation to a variety of foods (47, 48, 62). A recent study on the use of HHP on contaminated oysters and clams found that 300 MPa did not inactivate GI and GII noroviruses (63). Several studies on the use of HHP to inactivate surrogate viruses seeded in a variety of non-food matrices have been published (47, 62, 64-68). Unfortunately, a wide variety of
experimental conditions and a limited range of pressures to evaluate each virus were used which makes these results very difficult to compare across the different surrogate viruses. Because different inactivation conditions may impact virus inactivation by HHP (34), we employed the same test conditions for all viruses tested and used a range of 100 to 800 MPa for HHP. AiV is stable at 600 MPa for 5 min at 4°C to 21°C (68), and we confirmed these findings for 800 MPa at 4°C for 1 min. Inactivation of FCV and MNV was achieved at roughly similar MPa as reported previously (47, 64, 67). We found that MNV was more resistant than FCV which was completely inactivated at 300 MPa, whereas MNV required 400 MPa for complete inactivation. Kingsley also found that MNV was more resistant than FCV (34). Using HHP, near 4 log₁₀ reduction has been reported for TuV and 2 log₁₀ for MNV in culture medium at 21°C for 2 min using 350 MPa (62), while we found 3.5 log₁₀ reduction of each virus after exposure for 1 min at 4°C using 300 MPa. These differences clearly demonstrate the challenge to compare results from different studies. It is possible that differences in temperature (21°C vs 4°C in our study) contributed to these different findings. Using the appropriate pressure is critical for successful inactivation of human norovirus, as was shown in a study where human volunteers were fed raw oysters which had been artificially seeded with Norwalk virus and treated with HHP. Only HHP treatment at 600 MPa, and not 400 MPa, completely inactivated the virus and resulted in no infection in any of the subjects (45), which is comparable to the 600 MPa needed to completely inactivate Tulane virus in our study. PEC inactivation with HHP treatment has not been reported previously, but like MNV, PEC was also inactivated completely at 400 MPa.
Our study had several limitations. In order to compare the cultivable surrogate viruses with different inactivation conditions, which has not done before, we selected important test variables for each inactivation and disinfection condition. For example, we tested all viruses in cell culture medium; whereas a complex matrix such as food or fecal matter may provide different results. Inactivation of each surrogate virus dried on surfaces was studied only for two chlorine concentrations and one contact time, but since chlorine is commonly used at 200 ppm as a food contact sanitizer (59) and the use of 1,000 to 5,000 ppm is recommended to disinfect norovirus from non-food surfaces (69), our data mimic what is commonly used in food preparation environments. Although the results are promising, additional HHP treatments of surrogate viruses, perhaps mixed with human norovirus, seeded into various food matrices are needed to assess the practical application of HHP. Before considering round-robin testing with several laboratories, additional data are needed with selected surrogates such as TuV. This could provide a better understanding of which inactivation methods may provide optimum treatments for the best public health outcomes. Finally, we evaluated only selected treatment conditions for RNA reduction and a limited number of human norovirus genotypes. The observed differences in the sensitivity towards alcohol of GI.1 compared to GII.13 found in this study suggest that there may be more than one inactivation profile for human noroviruses and therefore additional genotypes should be tested.

Despite extensive efforts, to date all attempts to cultivate human norovirus in cell culture have failed (7-10). Consequently, the use of cultivable surrogate viruses is the approach available to most investigators to evaluate the effectiveness of control methods to prevent the spread of noroviruses and to protect public health. Our comprehensive study on comparing the...
performance of several key norovirus control measures, such as chlorine and alcohol for surface
and hand disinfection, demonstrates that depending upon which treatment is evaluated, the
choice of a surrogate virus for human norovirus could be narrowed down to one or two viruses.
However, ultimately the performance of the surrogate viruses will need to be linked to
reduction of infectivity of human noroviruses.

Because of the reported differences in susceptibility to different inactivation methods
for MNV and FCV, the validity of the use of surrogate viruses has been questioned and the use
of human challenge studies has been recommended to determine which techniques are
effective to reduce noroviruses in foods (70). This was nicely shown for HPP to determine the
appropriate pressure needed for complete inactivation of norovirus in oysters (45). However,
such studies are expensive and not allowed in many countries. But since data from clinical trials
provide us with the best possible data which intervention methods actually work, linking well-
designed surrogate studies with clinical trials in human volunteers may be the best approach
forward to reduce the number of norovirus outbreaks.

Although several molecular approaches for the detection of potentially infectious virus
have been evaluated as reviewed by Knight et al. (11), none are fully successful. Because
damage to the virus capsid is the only or primary virus damage inflicted by several of the
inactivation methods, methods that offer the most promise are those that rely on binding to an
intact viral capsid such as capture of virus by HBGA followed by detection of viral RNA by RT-
qPCR(71). Ultimately, a more basic understanding of the mechanisms of disinfection including
answers to questions such as which residues of viral capsid and/or genome are involved during
disinfection and whether these changes are similar across different enteric viruses will need to be answered (72). Such information could provide us with important insights on how to measure loss of infectivity of human noroviruses without an in vitro cell culture assay.

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REFERENCES


Table 1  Chlorine treatment of surrogate viruses dried on stainless steel discs

<table>
<thead>
<tr>
<th>Chlorine concentration</th>
<th>Log$_{10}$ reduction infectivity*</th>
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<tbody>
<tr>
<td></td>
<td>Virus type</td>
</tr>
<tr>
<td></td>
<td>AiV</td>
</tr>
<tr>
<td>200 ppm</td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td>1,000 ppm</td>
<td>1.3 ± 0.9</td>
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</table>

*Mean of 4 or more replicates from 2 separate experiments plus/minus the standard deviation. Exposure to 1,000 ppm inactivated FCV by 5 log$_{10}$ (p<0.0001), which was significantly higher than those of other three viruses that had less than 1-log reduction (P<0.001)

LEGENDS TO FIGURES

Figure 1. Reduction of virus infectivity after treatment for 30 min at 37°C at pH 2, 3, 9 or 10. Virus infectivity was measured by plaque assay (AiV, FCV, MNV and TuV) or by TCID$_{50}$ (PEC). Each experimental bar represents mean of 4 or more replicates with standard deviation shown by horizontal bars.

Figure 2. Stability of surrogate viruses at 56°C with increasing contact time. Virus infectivity was measured by plaque assay (AiV, FCV, MNV and TuV) or by TCID$_{50}$ (PEC).

Figure 3. Reduction of virus infectivity after treatment for 1 or 5 min with isopropanol or ethanol (70% or 90%): A, 70% for 1 min; B, 90% for 1 min; C, 70% for 5 min; D, 90% for 5 min. Virus infectivity was measured by plaque assay (AiV, FCV, MNV and TuV) or by TCID$_{50}$ (PEC).

Figure 4. Reduction of human norovirus RNA (GI.1, GI.5, GI.5 SP, GII.13 and GII.13 SP), MNV RNA, and MNV infectivity after exposure to 70% and 90% ethanol for 1 min.
Figure 5. Reduction of RNA from surrogate viruses (AiV, FCV, MNV, TuV) and human norovirus (GI.5 SP and GII.13 SP) after 200 or 1,000 chlorine treatment. Viruses were dried on stainless steel discs as described in Materials and Methods and then treated 5 min with chlorine.

Figure 6. Reduction of infectivity of surrogate viruses (AiV, FCV, MNV, PEC and TuV) after HHP treatment for 1 min at 4°C at varying MPa (100-800).

Figure 7. Reduction of TuV infectivity and TuV RNA and human norovirus RNA (GI.5 SP and GII.13 SP) after HHP treatment at 100-800 MPa for 1 min at 4°C. TuV infectivity was measured by plaque assay, and RNA reduction was calculated from control and treated samples measured by RT-qPCR.
Figure 1

Virus infectivity reduction (log_{10})

<table>
<thead>
<tr>
<th>pH 2</th>
<th>pH 3</th>
<th>pH 9</th>
<th>pH 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>AiV</td>
<td>FCV</td>
<td>MNV</td>
<td>TuV</td>
</tr>
<tr>
<td>PEC</td>
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</tbody>
</table>
Figure 2

Virus infectivity reduction (log_{10})
Figure 3

A 1 min

Virus reduction (log10) Isopropanol Ethanol

B 90%

Virus reduction (log10) Isopropanol Ethanol

C 5 min

70%

D

90%

Virus reduction (log10) Isopropanol Ethanol
Figure 4: Reduction of RNA and infectivity (log_{10})

- Ethanol 70% - Infectivity
- Ethanol 70% - RNA
- Ethanol 90% - Infectivity
- Ethanol 90% - RNA
Figure 6

Virus infectivity reduction (log_{10})

MPa

MPa

AiV
FCV
MNV
PEC
TuV

0
100 200 300 400 500 600 700 800

Virus infectivity reduction (log_{10})

MPa