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Stability of and Attachment to Lettuce by a Culturable Porcine Sapovirus Surrogate for Human Caliciviruses

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Human noroviruses (HuNoVs) are the leading cause of food-borne illness, accounting for 58% of U.S. cases. Because HuNoVs are unculturable, surrogates are needed to investigate transmission routes and evaluate disinfection methods. However, the current surrogates, feline calicivirus (FCV) and murine NoV (MNV), are less tolerant than HuNoVs to acid and chlorine, respectively. Porcine sapovirus (SaV) is the only culturable enteropathogenic calicivirus. In this study, the resistance of SaV to physicochemical treatments was compared to that of HuNoVs (by reverse transcription-PCR), FCV, and MNV (by infectivity assays). Sapovirus and HuNoV (viral RNA) showed similar resistances to heat (56°C) and to different concentrations of chlorine. However, SaV was more resistant than HuNoVs to ethanol treatment (60% and 70%). Like HuNoVs, SaV was stable at pH 3.0 to 8.0, with a <1.0 $\log_{10}$ 50% tissue culture infective dose ($TCID_{50}$) reduction at pH 3.0 compared to the value for pH 4.0 to 8.0. SaV and MNV showed similar resistances, and both were more resistant than FCV to heat inactivation (56°C). FCV was more resistant than MNV and SaV to ethanol, and all three viruses showed similar resistances to treatment with low concentrations of chlorine for 1 min. Those results indicate that SaV is a promising surrogate for HuNoVs. Next, we used SaV as a surrogate to examine virus attachment to lettuce at different pHs. Sapovirus attached to lettuce leaves significantly at its capsid isoelectric point (pH 5.0), and the attached viral particles remained infectious on lettuce after 1 week of storage at 4°C. The culturable SaV is a good surrogate for studying HuNoV contamination and transmission in leafy greens and potential disinfectants.

Human norovirus (HuNoV) is the leading cause of food-borne illnesses in the United States, accounting for 58% of all cases (46). This virus is highly contagious, with an estimated medium infectious dose of 18 viral particles (50). The virus titers are high during peak shedding, with a load of approximately 10 to 11 $\log_{10}$ genomic equivalents (GE)/g in stool samples (5, 6). Shedding can persist for weeks after the symptoms have resolved (48). About 20% of norovirus (NoV)-infected individuals do not show clinical signs (33), and asymptomatic individuals, such as food handlers, can be important sources of infection (30). Based on viral RNA stability, human NoVs are environmentally stable and resistant to many disinfectants, such as alcohol and certain antiseptic hand solutions (27, 40). These viruses display high genetic diversity, with over 25 genotypes within three genogroups (GI, -II, and -IV) (5, 55). Although short-term protective immunity exists for NoV infection based on volunteer studies (1), there is little evidence for cross-protection among genotypes, and susceptible individuals can be serially infected by different genotypes and may suffer from gastroenteritis after each infection (48).

A major obstacle to the study of HuNoVs is the lack of a routine cell culture system for assessment of virus infectivity. NoV-contaminated foods can meet bacteriological standards and still be distributed to the market (15). Also, there are no assays for direct evaluation of the inactivation efficiency for HuNoVs for food decontamination. This problem is partially overcome by using surrogate culturable caliciviruses. An ideal surrogate virus should be associated only with acute gastroenteritis, like HuNoVs, and have an environmental resistance similar to that of HuNoVs. One commonly used surrogate is murine NoV (MNV) (54). However, MNV infections show different clinical manifestations compared to HuNoVs. Although it replicates in the intestine and causes weight loss and diarrhea in immunocompromised mice, in general it also disseminates to multiple peripheral tissues and causes lethal systemic diseases, such as pneumonia and encephalitis, in immunocompromised mice and does not produce clinical symptoms in wild-type mice (35, 54). The other commonly used surrogate is feline calicivirus (FCV), belonging to the *Vesivirus* genus within *Caliciviridae* (45, 49). While HuNoVs are enteropathogenic viruses and acid stable (10), FCV is a respiratory virus and sensitive to low pH (11, 40). However, food processing and preservation conditions sometimes produce low pH. For example, acidic chlorine and chlorine-containing compounds are currently the most common methods of disinfection in the food industry in the United States. Acidic electrolyzed water was shown to have decontaminative effects and has been applied in the produce industry as an alternative method for disinfection (22). Therefore, neither MNV nor FCV is an ideal surrogate for HuNoVs. The cell culture-adapted porcine sapovirus (SaV) Cowden strain belongs to the *Sapovirus* genus within *Caliciviridae*. The wild-type Cowden strain causes gastroenteritis in gnotobiotic pigs. So porcine SaV is an alternative enteropathogenic calicivirus resembling HuNoVs and human SaVs (8, 14). After continuous passaging of the original porcine SaV in porcine primary kidney cells and then in a continuous swine kidney cell line, LLC-PK, the porcine SaV was adapted to cell culture and replicated to high infectious titers (6 to

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7 $\log_{10}$ 50% tissue culture infective doses [$TCID_{50}$]/ml; unpublished data) (7, 13, 42). The first aim of this study was to evaluate whether porcine SaV has a stability similar to that of HuNoVs (by reverse transcription [RT]-PCR), FCV, and MNV (by infectivity assays) after heat, chlorine, and alcohol treatments and can be used as an additional surrogate for HuNoVs. These modes of inactivation were chosen because heat is commonly used for food preparation and processing and in emergencies with water, chlorine is used to disinfect water and wash leafy greens as well as food surfaces and food contact surfaces, and alcohol is the major disinfection reagent for skin and surfaces. These inactivating agents are expected to represent different modes of attack: heat and alcohol principally attack the viral capsid proteins, and chlorine is thought to affect both capsid proteins and the RNA.

Food-borne-disease outbreaks associated with leafy greens are increasing in the United States (17). During 1986 to 1995 and 1996 to 2005, consumption of leafy greens (lettuce, spinach, and other greens) in the United States increased 17% and 9%, respectively. Meanwhile, leafy-green-associated food-borne-disease outbreaks increased 60% and 39%, respectively, much more than the increased rates of consumption. Most of the leafy green outbreaks with a confirmed etiology were caused by HuNoVs (58%). This reflects a convergence of increasing consumption of fresh produce, changes in production and distribution, a growing awareness of the problem on the part of public health officials (28), and changes in the human population, with more highly susceptible individuals worldwide because of aging, malnutrition, HIV infections, and other underlying medical conditions (http://www.who.int/mediacenter/factsheets/fs124/en/). Leafy greens may be contaminated pre- or postharvest at any point in the production chain. Lessons from numerous outbreaks indicate that contamination cannot be removed by simply washing the uncooked, ready-to-eat fresh produce (28). Thus, the prevention of contamination is vital. Further research on the mechanisms of contamination and decontamination using validated surrogates is required for development of improved prevention strategies.

Caliciviruses are nonenveloped viruses. So, viral particles, consisting of proteins, precipitate at the isoelectric point (pI) of the capsid protein and thus may behave uniquely at different pHs during virus attachment. The second aim of this study was to test whether porcine SaV attaches to lettuce leaves at different pHs and if the attached virus remains infectious after 1 week of lettuce storage at 4°C.

MATERIALS AND METHODS

Viruses and cell lines. The tissue culture-adapted porcine SaV Cowden strain was propagated in a porcine kidney cell line, LLC-PK (ATCC CRL-1392, currently ATCC CL-101). The LLC-PK cells were grown in minimal essential medium (MEM) containing 1% nonessential amino acids (NEAA), 1% antibiotics (10,000 units of penicillin, 10,000 μg of streptomycin, and 25 μg of amphotericin B/ml), and 5% fetal bovine serum (FBS). Propagation of porcine SaV in LLC-PK cells requires supplementation with 50 μM glycochenodeoxycholic acid (GCDCA; Sigma, St. Louis, MO) in maintenance medium without FBS as described previously (7). The SaV-infected cell culture supernatants had a titer of 6.20 ± 0.34 $\log_{10}$ $TCID_{50}$ as tested by immunohistochemistry (IHC) staining (see below) and were used for virus stability studies and lettuce experiments. The human NoV strains GII.4/HS194/2009/US (GenBank accession no. GU325839) and GII.g (RdRp)/GII.12 (capsid)/HS200/2010/US (abbreviations, GII.12/HS194 and GII.12/HS200, respectively) were detected in the stools of children with clinical gastroenteritis and were genetically characterized in our laboratory (48). The 1% or 10% fecal suspension in 0.01 M phosphate-buffered saline (PBS; pH 7.2) of HuNoVs was used for virus stability studies. The MNV S7 strain (GenBank accession no. AB435515) was a gift from Yukinobu Tohya at Nihon University, Japan (21). MNV was propagated in mouse leukemic monocyte macrophage cell line RAW 264.7 (ATCC TIB-71) with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS (heat inactivated at 60°C for 60 min), 1% antibiotics, 10 mM HEPES and 1 mM sodium pyruvate as described previously (21, 40). The culture supernatants of MNV had a titer of 5.68 ± 0.29 $\log_{10}$ $TCID_{50}$/ml by observation of cytopathic effects (CPE) and were used for virus stability studies. The FCV F9 strain was a gift from Louise Harris and was stored in our laboratory for 35 years. This strain was confirmed by RT-PCR with primers (forward, 5′-TGCTTTCC ACAAACTCAACC-3′, and reverse, 5′-CCCAGATCATCCTTCTTTTC C-3′) described by Park and Sobsey (41). Crandell Reese feline kidney (CRFK; ATCC CCL-94) cells were grown in MEM supplemented with 5% FBS, 1% NEAA, 1 mM sodium pyruvate, 1% antibiotics, and 10 mM HEPES. FCV was propagated in CRFK cells in medium with 2% FBS as reported previously (40). The culture supernatants of FCV had a titer of 7.29 ± 0.38 $\log_{10}$ $TCID_{50}$/ml by observation of CPE and were used in the virus inactivation studies. The FBS was from Thermo Scientific, and other cell culture reagents were from Invitrogen.

Titration of infectious porcine SaV, FCV, and MNV. Briefly, LLC-PK and CRFK cell monolayers and subconfluent RAW264.7 cells in 96-well plates were infected with serially diluted SaV, FCV, and MNV, respectively. The maintenance medium was supplemented with 50 μM GCDCA, 2% FBS, and 10% FBS for SaV, FCV, and MNV, respectively. The plates were incubated at 37°C for 3 days. For FCV and MNV, CPE caused by virus infection were observed directly under a light microscope. For SaV, infected cells were detected by IHC staining. LLC-PK cells were fixed with 3.7% formaldehyde in PBS at room temperature for 30 min, and then the fixed cells were permeabilized with 1% Triton X-100 in PBS at room temperature for 5 min. Guinea pig hyperimmune antiserum to virus-like particles (VLPs) of porcine SaV (16) and horseradish peroxidase (HRP)-conjugated goat anti-guinea pig IgG(H+L) (KPL, Gaithersburg, MD) were used as the primary and secondary antibodies, respectively. Finally, the substrate aminoethylcarbazole (AEC; AEC staining kit, Sigma) was used for color development. The cytoplasm of SaV-infected cells was stained red and observed using a light microscope. The virus titers were expressed as $TCID_{50}$ as the reciprocal of the highest sample dilution in which virus replicated in 50% of the wells (4 replicates) as calculated by the Reed-Muench method. Negative samples were assumed to have titers at the detection limit of 1.00 (for ethanol treatment) or 0.00 (for heat and chlorine treatment) $\log_{10}$ $TCID_{50}$/50 μl, which was used as the calculation baseline.

RNA extraction. RNA was extracted by using a 5× MagMAX-96 Viral 1 kit and a MagMax Express magnetic particle processor RNA/DNA extraction robot (Applied Biosystems, Foster City, CA) according to the manufacturer's manual.

Real-time RT-qPCR for detection of porcine SaV. A one-step TaqMan real-time quantitative RT-PCR (RT-qPCR) was developed for the detection of porcine genogroup III (GIII) SaVs. The forward primer (5′-CCA GAA GTG TTC GTG ATG GAG-3′; nucleotides [nt] 5125 to 5145 of the genome of the SaV Cowden strain [GenBank accession no. AF182760]), reverse primer (5′-GCC CRG CTG GYT GGA CTG-3′; nt 5230 to 5213 of the genome), and probe (5′,6-carboxyfluorescein [5′,6-FAM]–TGCGAGCAACCCAGAGGGCACTCA–Iowa Black fluorescence quencher [IABLFQ]; nt 5169 to 5192 of the genome) target the beginning of the major capsid VP1 gene. A plasmid DNA containing the sequence (the 3′-end 3 kb, including partial RNA-dependent RNA polymerase, VP1, VP2, and the 3′ untranslated region [UTR]) of the Po/SaV/GIII/OH-JJ259/00/US strain (GenBank accession no. AY826423) was used for the generation of a standard curve. The 20-μl RT-PCR mixture included 2 μl of sample RNA, 400 μM deoxynucleoside triphosphates (dNTPs), 200 nM each primer, and 100 nM the probe by using a

Qiagen OneStep RT-PCR kit (Qiagen, Valencia, CA). The reverse transcription was performed at 50°C for 30 min, followed by 95°C for 15 min, and the PCR was performed for 45 cycles (95°C for 15 s and 57.5°C for 60 s) on an Eppendorf Mastercycler RealPlex instrument (Eppendorf, Germany). The assay was sensitive, detecting ≥10 genomic equivalents (GE) per reaction (20 μl). It did not detect other genogroups of porcine SaVs (GVI/JJ681/00/US, GVII/OH-LL26/02/US, and GVIII/MI-QW19/02/US) (53) or porcine NoVs (GII.11/QW48, GII.18/QW101, and GII.19/QW170) (52).

RT-qPCR for detection of GII NoVs. The one-step TaqMan RT-qPCR assay for the detection of GII NoVs (using the primer set comprising COG2F [5′-CAR GAR BCN ATG TTY AGR TGG ATG AG-3′] and COG2R [5′-TCG ACG CCA TCT TCA TTC ACA-3′] and the probe RING2 [5′,6-FAM–TGG GAG GGC GAT CGC AAT CT–Black Hole quencher [BHQ]) was described by Kageyama et al. (19). The assay was performed by using the same OneStep RT-PCR kit and thermocycler as described for the SaV RT-qPCR. The detection limit of this assay is 10 GE per reaction (20 μl), as determined based on the standard curve generated using serially diluted plasmid DNA carrying Hu/NoV/GII.4/HS66/01/US (GenBank accession no. EU105469)-specific COG2F/2R amplicons.

IC RNA for detection of RT-PCR inhibitors. An internal-control (IC) RNA described previously (51), containing the vector pCR2.1-TOPO (nt 841 to 859; Invitrogen) and SaV partial RNA polymerase sequences, was used as an artificial template in the above-mentioned SaV or GII NoV RT-qPCR for a multiplex RT-qPCR to monitor the existence of RT-PCR inhibitors. Forward primer PECIC-F (5′-GCAAGCGCAAAGAGAAAGC AGGTA-3′) and reverse primer PEC-IC-R (5′-ATTCCGGTTCGCTTGC TGTCCATA-3′) gave a 91-bp amplicon. The probe PECIC-P (5′-Cy5-TTGCAGTGGGCTTACATGGCGATAGCTA-Iowa Black RQ) was labeled with Cy5, whose emission wavelength (650/670 nm) is distant from that of 6-FAM (521 nm), with which the SaV and GII NoV probes are labeled, to avoid potential interference among fluorescent dyes in a multiplex RT-qPCR. The amount of IC RNA added to each reaction mixture generated a threshold cycle (C_T) value of 28 to 29. The reaction was performed by using the SmartCycler system (Cepheid, Sunnyvale, CA). We considered an increase of ≥1.5 in C_T values for the IC RNA in the test sample compared to the values for the control samples an indication of weak inhibition (26).

Heat inactivation of porcine SaV and HuNoVs. The porcine SaV-infected cell culture supernatants (10 $\log_{10}$ GE/ml and 7 $\log_{10}$ $TCID_{50}$/ml) and a 10% fecal suspension of the human NoV/GII.4/HS194 strain (10 $\log_{10}$ GE/ml in PBS) were incubated at 56°C in a water bath for 30 min or 2 h and then placed on ice immediately. Control viruses were kept on ice during the experiment. The samples were stored at 4°C and tested within 2 days. Each RNA sample was tested in triplicate for RT-qPCR. Infectious SaV was titrated in duplicate by an IHC infectivity assay. Heat inactivation experiments were repeated twice more, with each experiment performed in triplicate.

Chlorine treatment of porcine SaV and HuNoVs. The HuNoV GII.4/HS194 and GII.12/HS200 strains were prepared as 1% stool suspensions in PBS and had an RNA titer of 9 $\log_{10}$ GE/ml as quantified by RT-qPCR. The SaV culture supernatants were diluted 1:10 in 1% human normal stool suspension in PBS to obtain a similar viral RNA titer, 9 $\log_{10}$ GE/ml (6 $\log_{10}$ $TCID_{50}$/ml). The commercial bleach (Clorox Co., CA), purchased locally, contained 5.7% sodium hypochlorite (NaOCl), corresponding to the typical free-chlorine level of 5 to 15%. Different concentrations of sodium hypochlorite (2.5, 10, 50, 100, and 200 mg/liter) were prepared in autoclaved Milli-Q water (room temperature) immediately before each experiment. Experiments were performed according to standard E1052 (http://www.astm.org) and the method of Macinga et al. (29). Briefly, 100 μl of virus solution was added to 900 μl of sodium hypochlorite solution or control water and mixed well. One hundred microliters from the water control tubes was sampled and stored at 4°C (time zero [T_0] samples). Then, the tubes were incubated at room temperature with rocking at low speed for 30 min. Finally, 80 μl of 25 mM sterile sodium thiosulfate was added to each tube to neutralize the chlorine. The samples were stored at 4°C and tested within 2 days. Each RNA sample was tested in triplicate for RT-qPCR. The SaV samples were diluted 10-fold in MEM prior to the IHC infectivity assay, which was performed in duplicate. The experiment was repeated twice more, with each experiment performed in triplicate.

Ethanol treatment of porcine SaV and HuNoVs. The human NoV/GII.4/HS194 strain was prepared as 10% stool suspensions in 1× PBS (pH 7.4) and titrated as 9 $\log_{10}$ GE/ml by RT-qPCR. The SaV-infected cell culture supernatants and its 1:10 dilution in normal human stool suspension in PBS were used in this experiment. The undiluted SaV had an infectious virus titer of 6 $\log_{10}$ $TCID_{50}$/ml, and the 10-fold-diluted SaV had an RNA titer of 9 $\log_{10}$ GE/ml. Two concentrations of ethanol (60% and 70%, vol/vol) were prepared in autoclaved Milli-Q water immediately before each experiment. Briefly, 20 μl of virus solution was added to 180 μl of ethanol solution or control water, mixed well, and incubated at room temperature for 5 min with rocking. Immediately, 10 μl of the treated virus was sampled and diluted 100-fold in PBS to dilute ethanol to final concentrations of 0.6% and 0.7%. The samples were stored at 4°C and tested within 2 days. Each RNA sample was tested in triplicate by RT-qPCR. The infectious SaV was titrated in duplicate in the IHC infectivity assay. The experiment was repeated twice more, with each experiment performed in triplicate.

Heat, ethanol, and chlorine inactivation of porcine SaV, FCV, and MNV. Porcine SaV and FCV were diluted 1:2.5 and 1:50, respectively, in PBS (0.01 M, pH 7.2) supplemented with FBS to get the same infectious titer (5.70 $\log_{10}$ $TCID_{50}$/ml) and final FBS concentration (10%) as those of the MNV culture supernatants. For heat inactivation, viruses were incubated at 56°C in a water bath for 30 min or 2 h and then placed on ice immediately. Control viruses were kept on ice during the experiment. For ethanol inactivation, 1 volume of virus solution was added to 9 volumes of ethanol solution (60% or 70%) or control water, mixed well, and incubated at room temperature for 30 s. Immediately, the treated virus was serially diluted 10-fold (5.4% or 6.3% ethanol) and 100-fold (0.54% or 0.63% ethanol) in PBS to dilute ethanol to avoid further inactivation. Samples from 1:10 and 1:100 dilutions were tested. For chlorine treatment, 1 volume of virus solution was added to 9 volumes of sodium hypochlorite (2.5 mg/liter or 10 mg/liter) or control water, mixed well, and incubated at room temperature for 1 min. Immediately, sodium thiosulfate was added to each tube to neutralize the chlorine. The treated samples were titrated on the same day that the experiment was performed. Comparative inactivation studies of the three viruses were performed three times, with each experiment performed in triplicate.

pH treatment of porcine SaV and impact of pH on attachment of porcine SaV to lettuce leaves. Romaine lettuce (*Lactuca sativa*) was purchased from a local grocery store. Intact lettuce leaves without visible damage were washed with tap water first and then washed with autoclaved sterile water once. The washed leaves were put on paper towels to dry for 30 min. Buffers of various pHs (3.0, 4.0, 5.0, 6.0, 7.0, and 8.0) were prepared as follows. Phosphate-citrate buffers (0.2 M dibasic sodium phosphate, 0.1 M citric acid) were prepared for solutions of pH 3.0, pH 4.0, and pH 5.0, and sodium phosphate buffers (0.1 M, different ratios of the mono- and di-basic sodium phosphate) were prepared for solutions of pH 6.0, pH 7.0, and pH 8.0. The buffers were filtered through 0.22-μm-pore-size filters before the experiments. Lettuce leaves were cut into small pieces (4 by 6 cm^2 per piece) and placed into separate Whirl-pak plastic bags (591-ml size) containing 20 ml of buffer. Each pair of samples included the virus+buffer+lettuce group, consisting of 100 μl of SaV-infected culture supernatants diluted in buffer (final concentration, 3.7 $\log_{10}$ $TCID_{50}$/ml) containing lettuce, and the virus+buffer group, consisting of virus in buffer without lettuce (virus control). Lettuce in buffer without virus was tested as a mock control to test whether the samples were toxic to cells in the IHC infectivity assay. The bags were rocked at low speed at room temperature for 1 h to allow virus binding. Then, 1 ml of solution was removed from each bag for SaV infectivity assays. Virus-inoculated let-

TABLE 1 Reductions in SaV and HuNoV RNA after physicochemical treatments

Treatment	Conditions	Log_{10} reduction in GE (mean ± SD)	
		SaV	NoV/GII.4/HS194
Heat	56°C, 30 min	0.10 ± 0.12	0.16 ± 0.20
	56°C, 2 h	0.35 ± 0.07	0.53 ± 0.12
Ethanol	60%, 5 min	0.06 ± 0.10	1.51 ± 0.15[a]
	70%, 5 min	0.02 ± 0.04	1.37 ± 0.32[b]

[a] Significantly higher than the value for SaV after 60% ethanol treatment for 5 min ($P < 0.05$; ANOVA).
[b] Significantly higher than the value for SaV after 70% ethanol treatment for 5 min ($P < 0.05$; ANOVA).

tuce leaves that were incubated in pH 5.0 buffer were removed from the bags to sterile petri dishes, dried in a biohazard hood for 1 h, and then stored at 4°C for 1 week. Virus was eluted by repeated pipetting (25 times) using 4 ml of elution buffer (MEM plus 2% FBS) and tested by the IHC infectivity assay. Seven independent experiments, with 3 replicates per experiment, were performed ($n = 21$).

Data analyses. One-way analysis of variance (ANOVA) was performed to test whether infectious virus titers or viral RNA levels differed significantly. For the lettuce experiments, an exact binomial test based on binomial distribution was performed to determine whether binding of virus to lettuce was significant. We considered binding of virus to lettuce statistically significant when the binding proportion and 95% confidence level were both >50%. All statistical analyses were performed by using the Statistical Analysis System (SAS; version 9.2). A P value of <0.05 was considered significant.

RESULTS

Porcine SaV and HuNoV GII.4/HS194 had similar stabilities at high temperatures (56°C) as tested by RT-qPCR. The 56°C level is considered the lower temperature limit for "hot bars" (4). After incubation at 56°C, the RNA levels of porcine SaV and HuNoVs did not change after the 30-min treatment compared to the levels for the controls (Table 1) but differed significantly after the 2-h treatment ($P < 0.05$), with a <1.0 log_{10} reduction in both viruses. However, 30-min and 2-h treatments reduced infectious SaV by 2.38 ± 0.18 and >4.00 ± 0.53 log_{10} $TCID_{50}$, respectively (Table 2). Therefore, RT-qPCR results underestimated the efficacy of heat inactivation of SaV. This is not unexpected, because RNA is very heat stable, and temperatures of 95°C or higher are sometimes used as a means of extracting RNA from viruses.

Porcine SaV was more resistant than HuNoV to ethanol treatment. The porcine SaV and human NoV/GII.4/HS194 strains were treated with two commonly used concentrations of ethanol (60% and 70%, vol/vol) at room temperature for 5 min, mimicking the practical contact time for ethanol/alcohol disinfection. To avoid the potential further virus inactivation and cell toxicity caused by ethanol, the treated samples were further diluted 100-fold immediately after experiments. We did not detect RT-PCR inhibitors in the ethanol-treated and further-diluted samples by using the multiplex RT-qPCR with the IC RNA. Compared to those for the water control (5.29 ± 0.08 log_{10} GE/ml), the SaV RNA titers remained at the same level after 60% and 70% ethanol treatments. However, HuNoV HS194 RNA showed 1.51 ± 0.15 and 1.37 ± 0.32 log_{10} reductions after 60% and 70% ethanol treatments, respectively, compared to the water control (5.23 ± 0.12 log_{10} GE/ml). The SaV samples did not show any cell toxicity in LLC-PK cells. However, when we used the SaV diluted in normal human fecal suspensions in this experiment, infectious SaV titers in controls were 10- to 100-fold lower than expected (1.69 ± 0.42 log_{10} $TCID_{50}$/ml). The same phenomenon occurred when another normal human fecal suspension was used to dilute the SaV. So we suspected that the normal human fecal suspensions contained SaV infectivity inhibitory factors. To determine the reduction in infectivity, the experiments were repeated with the undiluted SaV culture supernatants. The SaV (2.93 ± 0.30 log_{10} $TCID_{50}$/ml) became undetectable (<1.3 log_{10} $TCID_{50}$/ml, a >1.63 log_{10} reduction) after 60% or 70% ethanol treatment for 5 min. These results indicated that SaV was more resistant than HuNoVs to ethanol treatment based on viral RNA levels and that RT-qPCR results underestimated the efficacy of ethanol disinfection of SaV (Table 1).

Porcine SaV and HuNoVs had similar resistances to chlorine treatments. The porcine SaV and HuNoV GII.4/HS194 and GII.12/HS200 strains were treated with different concentrations of sodium hypochlorite (2.5, 10, 50, 100, and 200 mg/liter) at room temperature for 30 min. The three concentrations (2.5, 10, and 200 mg/liter) were included because these are similar to the concentrations of free chlorine used for drinking water (WHO standard, 2 to 3 mg/liter; maximum, 5 mg/liter) and postcontamination treatment of water (10 mg/liter) and the maximum chlorine concentration used to disinfect items that may be put into the mouth, such as toys (200 mg/liter) (guidelines for environmental cleaning and disinfection of NoV [2009]; Michigan Department of Community Health and Michigan Department of Agriculture).

TABLE 2 Reductions in infectious FCV, MNV, and SaV after physicochemical treatments

Treatment	Conditions	Log_{10} reduction in $TCID_{50}$ (mean ± SD)		
		FCV	MNV	SaV
Heat	56°C, 30 min	>4.92 ± 0.16	2.42 ± 0.67[a]	2.38 ± 0.18[a]
	56°C, 2 h	>5.01 ± 0.25	>3.90 ± 0.43	>4.00 ± 0.53
Ethanol	60%, 30 s	0.75 ± 0.20	1.79 ± 0.46[b]	>2.22 ± 0.40[b]
	70%, 30 s	0.91 ± 0.27	>2.81 ± 0.38[c]	>2.22 ± 0.40[c]
Sodium hypochlorite	2.5 mg/liter, 1 min	0.52 ± 0.20	0.31 ± 0.23	0.26 ± 0.29
	10.0 mg/liter, 1 min	0.58 ± 0.13	0.87 ± 0.14	1.22 ± 0.65

[a] Significantly lower than the value for FCV after treatment at 56°C for 30 min ($P < 0.05$, ANOVA).
[b] Significantly higher than the value for FCV after 60% ethanol treatment ($P < 0.05$, ANOVA).
[c] Significantly higher than the value for FCV after 70% ethanol treatment ($P < 0.05$, ANOVA).

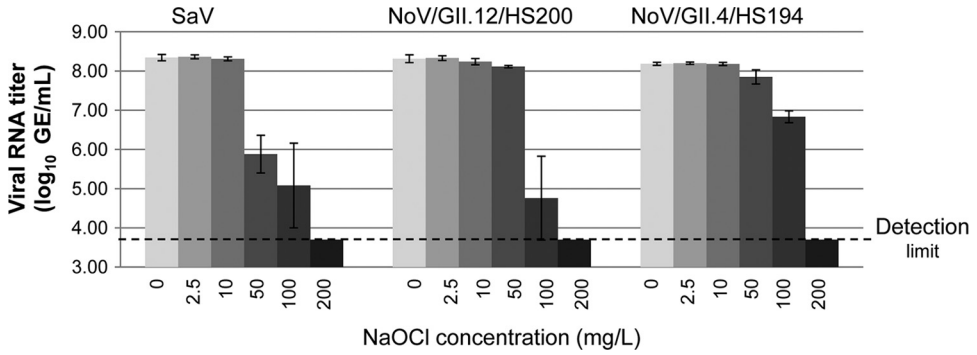

FIG 1 Comparison of porcine SaV and HuNoV RNA titers after treatment with different chlorine concentrations. The porcine SaV and HuNoV GII.4/HS194 and GII.12/HS200 strains were incubated with different concentrations of sodium hypochlorite (2.5, 10, 50, 100, and 200 mg/liter) or a water control at room temperature for 30 min. The treatment was stopped by adding sodium thiosulfate. Viral RNA was titrated by RT-qPCR assays. Data are the geometric mean titers, and error bars represent 95% confidence intervals (CI) from three experiments, with each experiment performed in triplicate.

We did not detect RT-PCR inhibitors in the chlorine-treated and sodium thiosulfate-neutralized samples by using the multiplex RT-qPCR with the IC RNA. SaV and HuNoV GII.12/HS200 and GII.4/HS194 RNA became undetectable only after sodium hypochlorite concentrations were increased to 200 mg/liter (Fig. 1). However, the cell culture infectivity assay showed that SaV lost infectivity (undetectable; <2.3 $\log_{10}$ $TCID_{50}$/ml, a >2.7 $\log_{10}$ reduction) at the lowest concentration (2.5 mg/liter) of sodium hypochlorite. The control samples did not show any cell toxicity to LLC-PK cells. Therefore, the RT-qPCR results underestimated the efficacy of chlorine disinfection of SaV. This has also been recognized previously for other RNA viruses (38, 39).

Porcine SaV, FCV, and MNV showed different resistances to different physicochemical treatments. To balance the potential protective effects of 10% FBS and of high concentrations of viruses, porcine SaV and FCV were diluted 1:2.5 and 1:50, respectively, in PBS (0.01 M, pH 7.2) supplemented with FBS to get the same infectious titer (5.70 $\log_{10}$ $TCID_{50}$/ml) and final FBS concentration (10%) as those of MNV culture supernatants. First, the resistances of the three viruses to heat treatment (56°C) were compared in parallel. After incubation at 56°C for 30 min, MNV and SaV were detected at reduced titers (2.42 ± 0.67 and 2.38 ± 0.18 $\log_{10}$ reductions, respectively) compared to controls (kept on ice), and FCV was almost undetectable, except for one of the three sample replicates of one experiment, which was weakly positive (0.80 $\log_{10}$ $TCID_{50}$/50 μl). After 2 h of incubation at 56°C, the infectious viral titers of all three viruses became undetectable (<0.00 $\log_{10}$ $TCID_{50}$/50 μl). The $\log_{10}$ reductions for FCV, MNV, and SaV were $>5.01 \pm 0.25$, $>3.90 \pm 0.43$, and $>4.00 \pm 0.53$, respectively, compared to the levels for the controls. These results indicated that SaV and MNV have similar resistances and that both viruses are more resistant than FCV to heat inactivation at 56°C (Table 2).

Next, we tested the resistance of SaV, FCV, and MNV to ethanol treatments (60% and 70%). We found that 1:10 and 1:100 dilutions of treated samples did not cause CPE on the LLC-PK (for SaV) and CRFK (for FCV) cell monolayers. However, RAW264.7 cells (for MNV) showed slower cell growth than mock controls, probably due to the ethanol in the 1:10 (5.4% and 6.3% ethanol) and 1:100 (0.54% and 0.63% ethanol) diluted samples, but it seemed that these did not interfere with MNV replication. When experiments were performed at room temperature for 5 min, as we did for the comparative study of SaV and HuNoV, infectious SaV and MNV became undetectable and FCV was detected occasionally at very low levels (1.00 to 1.33 $TCID_{50}$/50 μl). Thus, the incubation time was shortened to 30 s. After 60% ethanol treatment for 30 s at room temperature, SaV became undetectable, MNV was detected at low levels (2.02 ± 0.76), and FCV had a minor $\log_{10}$ reduction of 0.75 ± 0.20 (Table 2). Both SaV and MNV became undetectable when the ethanol concentration increased to 70%, but FCV still had a minor $\log_{10}$ reduction of 0.91 ± 0.27. The $\log_{10}$ reductions of SaV and MNV were comparable but significantly greater than those of FCV following treatments with both 60% and 70% ethanol concentrations ($P < 0.05$). These results indicated that FCV was more resistant to ethanol treatment than MNV and SaV.

Finally, we compared the resistances of SaV, FCV, and MNV to chlorine treatments (2.5 mg/liter and 10 mg/liter of NaOCl). Only two lower concentrations of sodium hypochlorite were chosen, and treatment was performed for a very short period of time (1 min) because SaV became undetectable after treatment with 2.5 mg/liter of NaOCl for 30 min, when SaV was compared with HuNoVs. We found that infectious FCV and MNV titers decreased significantly after 2.5 mg/liter or 10 mg/liter of NaOCl treatment, and SaV titers decreased significantly after 10 mg/liter of NaOCl treatment compared to the levels for the controls ($P < 0.05$). The three viruses showed similar $\log_{10}$ reductions in virus titer following treatment with the two concentrations of NaOCl (Table 2), indicating similar resistances to low concentrations of chlorine.

Binding of porcine SaV to lettuce leaves was significant at pH 5.0, and porcine SaV was stable at low and high pHs. To investigate whether porcine SaV binds to lettuce surface depending on pH, we incubated lettuce pieces and SaV in buffers with different pHs (3.0 to 8.0) in Whirl-pak bags at room temperature for 1 h. Meanwhile, we tested the stability of SaV at pH 3.0 to 8.0. We found that at each pH, except for pH 7.0, the overall SaV titer of the virus+buffer group was higher than that of the virus+buffer+lettuce group (Table 3). For each pair of samples, the virus titer of the virus+buffer group was higher than, equal to, or lower than that of the virus+buffer+lettuce group, due to whether the virus was bound to lettuce or not and experimental errors. Viruses in the virus+buffer+lettuce group remained in the buffer or attached to the lettuce or to the bag, whereas viruses in the virus+buffer group remained in the

TABLE 3 Infectious SaV titers after incubation at different pHs at room temperature for 1 h

Group	Titer ($\log_{10}$ $TCID_{50}$/ml) (mean ± SD)[b] after incubation at:					
	pH 3.0	pH 4.0	pH 5.0	pH 6.0	pH 7.0	pH 8.0
Virus+buffer+lettuce[a]	3.35 ± 0.54	3.97 ± 0.44	4.05 ± 0.52	4.14 ± 0.56	4.15 ± 0.63	4.09 ± 0.60
Virus+buffer	3.56 ± 0.48***	4.03 ± 0.45**	4.32 ± 0.48*	4.21 ± 0.50*,**	4.04 ± 0.54**	4.18 ± 0.50*,**

[a] The initial virus concentration was 3.7 $\log_{10}$ $TCID_{50}$/ml.
[b] For the virus+buffer group, titers with different numbers of asterisks differ significantly ($P < 0.05$; ANOVA and Tukey multiple comparison procedures).

buffer or attached to the bag. If the virus titer in the solution of the virus+buffer+lettuce group was lower than that of the virus+buffer group, this indicated binding of virus to lettuce. If the virus titer of the virus+buffer+lettuce group was higher than that of the virus+buffer group, this indicated no binding of virus to lettuce. Finally, if the virus titer of the virus+buffer+lettuce group was equal to that of the virus+buffer group, half of the replicates were considered binding or nonbinding in statistical analysis. After statistical analysis, we found that significant binding of SaV to lettuce occurred exclusively at pH 5.0, whose binding proportion and 95% confidence level were both >50% ($P < 0.0002$) (Table 4). After storage at 4°C for 1 week, the lettuce pieces still appeared fresh. The elution from the stored lettuce pieces that were incubated in pH 5.0 tested positive (80 to 184 $TCID_{50}$/24 cm^2 lettuce) by IHC infectivity assays.

Finally, samples from the virus+buffer group were also used to evaluate SaV stability at different pHs. After incubation of SaV (3.7 $\log_{10}$ $TCID_{50}$/ml) in buffers at different pHs at room temperature for 1 h, a <1.0 $\log_{10}$ difference of virus titers was seen among pHs (Table 3). Viral titers at pH 5.0 and pH 3.0 were significantly higher and lower than those at pH 4.0 and pH 7.0, respectively ($P < 0.05$). There were no significant differences in virus titers among buffers at pH 4.0, 6.0, 7.0, and 8.0.

DISCUSSION

Human NoVs cause an estimated 23 million cases of illness annually in the United States (32). NoV outbreaks often occur in confined community settings such as day care centers, schools, cruise ships, military bases, hospitals, and nursing homes. These outbreaks often result in facility closures due to the lack of effective disinfectants for NoVs and vaccines or antivirals, causing enormous economic impacts within such communities. For example, costs associated with a single NoV outbreak in a U.S. tertiary care hospital were estimated to be $657,644 (18). Although NoV infection is usually self-limiting, it can cause persistent infection for months and even death among the young, the elderly, and immunocompromised patients. Recently, CDC reports indicated that NoVs are the second and the fourth leading causes of food-borne-disease-associated hospitalization (26%) and deaths (11%), respectively, in the United States (46).

Porcine SaV is a promising surrogate for HuNoVs and can be used in addition to the current surrogates FCV and MNV. It is hard to test the anti-HuNoV efficacy of disinfectants, such as food decontamination methods and antiseptic hand solutions. This is partially because HuNoVs are unculturable and thus no cell culture infectivity assay is available. Several culturable-but-not-ideal viruses have been used as NoV surrogates to study virus disinfection, such as bacterial phage MS2, FCV, and MNV (3, 4, 11, 40, 43). These viruses showed different resistances to physicochemical treatments compared to HuNoV, and thus they may not be representative of HuNoVs for studying virus contamination and disinfection. By using RT-qPCR assays, we found that SaV and HuNoV had resistance to high temperature (56°C) and to different concentrations of chlorine (Table 1 and Fig. 1). Like HuNoVs (10), porcine SaV was resistant to low and high pHs, showing <1.0 $\log_{10}$ infectious dose reductions at pH 3.0 to 8.0 (Table 3). However, compared to HuNoV, SaV was more resistant to 60% and 70% ethanol treatments (Table 1). Simultaneous comparison of infectious SaV, FCV, and MNV showed that porcine SaV and MNV were more resistant to heat inactivation (56°C for 30 min or 2 h) than FCV, FCV was more resistant to ethanol treatment (60% or 70% for 30 s) than MNV and SaV, and the three viruses showed similar resistances to treatment with low concentrations of chlorine (2.5 or 10 mg/liter NaOCl for 1 min). Our results showing that FCV was more stable than MNV to alcohol were in agreement with previous reports (40). MNV was reported to be more stable than FCV in water at room temperature (2, 4). However, our results regarding the relative resistances of FCV and MNV to heat at 56°C were in opposition to those reported previously, in which it took 3.5 min and 6.7 min for MNV-1 and FCV, respectively, to achieve 1 log inactivations (4), indicating that FCV was more stable than MNV-1 to heat at 56°C. This discrepancy could be due to the different MNV strain (S7 strain) or the different period of incubation time (30 min and 2 h) that we used. Park and Sobsey (41) reported that FCV was more sensitive than MNV to 5,000 mg/liter (or ppm) NaOCl treatment. We did not see any differ-

TABLE 4 Numbers of replicates showing various results at different pHs[a]

Result[b]	No. of replicates ($n = 21$) showing result at:					
	pH 3.0	pH 4.0	pH 5.0	pH 6.0	pH 7.0	pH 8.0
No. in virus+buffer group > no. in virus+buffer+lettuce group (binding)	11	11	17	9	7	10
No. in virus+buffer group = no. in virus+buffer+lettuce group	0	3	3	2	1	2
No. in virus+buffer group < no. in virus+buffer+lettuce group (no binding)	10	7	1	10	13	9

[a] Seven experiments, with three replicates per experiment.
[b] The binding proportions (95% confidence levels) were as follows: for pH 3.0, 52% (30 to 74%); for pH 4.0, 60% (36 to 81%); for pH 5.0, 88% (68 to 99%) (significantly higher than 50%, indicating significant binding [$P < 0.0002$; exact Binomial test]); for pH 6.0, 48% (26 to 70%); for pH 7.0, 36% (15 to 59%); and for pH 8.0, 52% (30 to 74%).

ence between FCV and MNV, but experiments with higher concentrations and longer periods of incubation time should be performed to make final conclusions. Nevertheless, we suspect that none of the three viruses can fully recapitulate the features of HuNoV stabilities to different physicochemical treatments. Among the three viruses, MNV is the only norovirus and porcine SaV is the only enteropathogenic calicivirus. FCV grows to ~ 2 $\log_{10}$-higher infectious titers than MNV and SaV. When a disinfection method is evaluated, such a high infectious titer of FCV enables us to obtain a larger range of reductions in infectivity than the use of MNV or SaV. Because different genogroups or genotypes of HuNoVs have slightly different environmental stabilities (25), several surrogates, including viruses with stabilities identical to or slightly higher than those of tested human GII NoVs, should be tested to evaluate a disinfection method. The higher stability of porcine SaV than of FCV to heat and acid and the fact that SaV causes acute gastroenteritis in young pigs make this virus a promising surrogate for studying the transmission and inactivation of HuNoVs.

RT-qPCR tends to underestimate disinfection efficacy compared to infectivity assays. This drawback of RT-qPCR for virus disinfection studies has been recognized by many researchers (43). This is because most disinfection agents act on the capsid that protects the viral RNA, and in many cases, inactivation has little or no effect on the viral RNA. For example, certain inactivation treatments, such as pasteurization, cause little or no damage to the viral genome (44). Although the receptor ligands on a virion surface are damaged by the treatments, resulting in no infectivity, the virus particle may still be intact and viral RNA is protected within such a noninfectious viral particle, which can be detected by RT-PCR. Even for treatments that cause genome damage, such as UV-induced photodimers, a mutagenic lesion may be sufficient to cause virus inactivation. Because real-time PCR usually targets <150-nt fragments of the viral genome, it likely underestimates the inactivation efficiency. For a virus with a genome size of about 8,000 nt, such as NoV and SaV, if we assume that a minimum difference of 0.1 to 0.5 $\log_{10}$ in RNA titer is needed to exceed the signal-to-noise threshold, a single RT-PCR with an amplicon size of 500 to 1,000 nt would be needed or multiple short sections across different areas of the viral genome should be targeted by real-time PCR assays to estimate infectivity (43). This can explain why a >4.00 ± 0.53 $\log_{10}$ reduction by infectivity assay corresponded to a 0.35 ± 0.07 $\log_{10}$ reduction by the RT-qPCR assay for SaV after heat inactivation treatment for 2 h (Tables 1 and 2). Although porcine SaV and HuNoVs have similar genomic sizes, and the RT-qPCR assays for GIII SaV and GII NoVs target similar regions of the viral genome, only when SaV and NoV capsids have similar stabilities following a treatment can we use RT-qPCR assays to compare their stabilities.

Other methods used to distinguish infectious and inactivated HuNoVs. An infectious virus particle should possess an intact viral RNA genome, and its capsid should maintain its integrity to protect the RNA and attach to the receptor of a cell to initiate infection. For the unculturable HuNoVs, other methods that can detect RNA from intact viral particles have been suggested, such as using histoblood group antigen (HBGA)-like oligosaccharide-coated immunomagnetic beads to bind intact viral particles prior to RNA extraction (5). However, this approach is very challenging because it is unknown whether different genogroups/genotypes of HuNoVs use the same cell receptor, and some NoVs fail to bind HBGAs (48). Other reports described the successful use of proteinase K and/or RNase to treat samples prior to lysis of virus during RNA extraction to differentiate infectious and noninfectious viral particles (24, 36, 39). For unculturable viruses, this is still a valid approach for evaluating disinfection efficiency. However, a weakness is that some disinfectants may cause loss of the ability of a virion to attach to host cell receptors without loss of the protective function of the capsid (37).

Virions precipitate at the pI of the viral capsid protein to form aggregates that may facilitate binding of virus to lettuce and mitigate virus inactivation in the environment. The initial attachment of virus to surfaces is a key step in contamination. It was hypothesized that the relation between the isoelectric point (pI) of a virus (which ultimately depends on the amino acid capsid composition) and the pH of the surrounding medium will determine virus attachment to a surface if viruses do not use specific cell surface receptors to attach. The electroneutrality near the pI should theoretically favor adsorption (9). The calculated pI of the capsid protein of porcine SaV is pH 5.4. In our lettuce experiments (Table 4), SaV bound significantly to lettuce leaves around its pI, at pH 5.0, but not at pH 3.0, 4.0, 6.0, 7.0, and 8.0. The results suggest that near the pI, virions probably precipitate to form aggregates that may facilitate binding of virus to lettuce. HuNoVs and SaVs have similar calculated pIs (porcine SaV Cowden strain, pI 5.4; HuNoVs, pI 5.6 to 5.9) based on the capsid protein sequences. Therefore, our results for binding of porcine SaV to lettuce surfaces may reflect the HuNoV binding pattern. Recently, our group found that HuNoV (GII.4) VLPs bind to lettuce cell wall materials by utilizing multiple carbohydrate moieties (12). These carbohydrate-driven interactions, which are different from the nonspecific binding due to the surface charge of virions, are specific. Therefore, we believe that the overall binding of virus particles to lettuce is the result of both specific and nonspecific factors.

In our pH treatments of SaV, the viral titer at pH 5.0 was higher than those at other pHs. We suspected that the virus formed aggregates at pH 5.0 and that the virus particles inside these aggregates were protected from environmental inactivation. In a recent report (47), HuNoVs were more susceptible to chlorine treatment when the virus inoculum was purified and dispersed prior to inoculation into water than when the inoculum was not purified, in which case the viruses were more likely aggregated and/or particle associated (11, 20). These results suggest that aggregated and/or matrix-associated viruses are more resistant to environmental inactivation than individual viral particles.

Porcine SaV remains infectious on lettuce leaves after storage at 4°C for 1 week. The result obtained for porcine SaV is not surprising, because several studies have shown that viruses spiked on the lettuce surface can remain infectious for at least 1 week after storage at refrigeration conditions: infectious FCV was recoverable until day 7 (31), Kurdziel et al. (23) reported that it took 11.6 days for poliovirus to show a 1 $\log_{10}$ reduction in infectious titers, and coronavirus remained infectious for at least 14 days (34). One week in the refrigerator is the general storage time for lettuce. Contaminated lettuce could be an important vehicle for enteric virus transmission. Therefore, the initial prevention of contamination is vital for prevention of food-borne NoV or SaV outbreaks.

In conclusion, porcine SaV and HuNoVs have similar resistances to low pH, high temperatures, and chlorine, and SaV is

more resistant than HuNoVs to ethanol, based on RT-qPCR results. Porcine SaV and MNV were more resistant than FCV to heat (56°C) inactivation, FCV was more resistant than MNV and SaV to ethanol treatment, and the three viruses showed similar resistances to treatment with low concentrations of chlorine for a short time (1 min). It is likely that none of the three viruses can fully recapitulate the characteristics of HuNoV stabilities following different physicochemical treatments. Because SaV is the only enteropathogenic virus among the three viruses, SaV is a promising additional surrogate for studying the transmission and inactivation of human NoVs. The SaV bound significantly to lettuce leaves at its capsid pI (pH 5), and SaV-contaminated lettuce leaves retained infectious virus up to at least 1 week at 4°C. Contaminated lettuce is an important vehicle for enteric calicivirus transmission.

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