Efficacy of Sanitizing Treatments for Feline Calicivirus as a Norovirus Surrogate Attached to Food and Food Contact Surfaces

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Abstract

Norovirus (NV) is becoming a major cause of foodborne illness in many countries. At present, very little is known about the survival of NV in the environment or the disinfection procedures needed to remove NV from contaminated surfaces. Feline calicivirus (FCV, $1 \times 10^{6.75}$ $TCID_{50}$/mL) was used as a surrogate model for NV to investigate the effectiveness of sanitizing treatments for the viruses attached to food and food contact surfaces. Ammonium chloride (2%), organic acids (3000 ppm), and ethanol (70%) were most effective, providing 4 log_{10} (99.99%) reductions in FCV titers on food or food contact surfaces. The disinfection efficacies of most agents on ceramic and glass surfaces were greater than stainless steel. The results from this study can be applied in the food industry to reduce NV-associated foodborne illnesses.

Key words: norovirus, feline calicivirus, inactivation, $TCID_{50}$, disinfectants

INTRODUCTION

Many countries now consider foodborne disease to be a major ongoing public health issue. In recent years, it has been recognized that viruses are an important cause of foodborne disease. Unlike bacteria, viruses do not multiply in or on foods, but foods may become contaminated with human viruses and transmit infection. Foodborne viruses include rotaviruses, adenoviruses, caliciviruses, norovirus (NV), and hepatitis A virus (HAV) (1-3). Although, there are numerous varieties of viruses transmitted via the fecal-oral route, most reports of foodborne transmission involve NV and HAV infections, suggesting that these two strains are associated with a greater risk of foodborne transmission. NV and HAV can be transmitted from person to person or indirectly, via food, water, or virus-containing feces or vomit. Characteristic symptoms of nausea, vomiting, and diarrhea typically appear after 24~48 hr of incubation and last about 48~72 hr (4). Most individuals recover completely without complications, but the very young, the elderly, and persons with weakened immune systems may require special care (2,3,5,6). Transmission of these viruses by contaminated foods, especially oysters, vegetables, fruits, and water, has been documented (2,7,8). In recent years, the incidence of NV food poisoning has increased rapidly in Korea. According to a report by the Division of Enteric Hepatitis Viruses, about 5.5% of acute diarrhea cases and 30% of viral diarrhea cases between 2003

and 2007 were due to NV. The Korean Food and Drug Administration reported that 97 of 510 foodborne outbreaks and 2345 of 9686 foodborne illnesses that occurred in 2007 were caused by NV (9). Recent advances in NV research have led to the development of methods that can be used to trace and detect viral strains. These methods can and have been used for the detection of common source outbreaks (2,3,6,7,10,11); however, very little is known about the survival of NV in the environment or the disinfection procedures needed to remove NV from contaminated settings. Food contact surfaces have not been investigated for their role in the transmission of NV, possibly because effective methods are not available for virus recovery from such surfaces. Consequently, it is necessary to assess disinfection protocols and the survival of NV in the environment. NV cannot be grown in cell culture; thus, cultivable feline calicivirus (FCV) was used as a surrogate model (4,5, 7,10-24). FCV and NV belong to the same family of *Caliciviridae* and their genetic and morphological properties are very similar. The objective of this study was to develop methods to recover FCV from food and food contact surfaces. We then evaluated several disinfectants for their ability to inactivate FCV.

MATERIALS AND METHODS

Food contact surfaces
The food contact surfaces were made of stainless steel,

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glass, and ceramic. Stainless steel (type 304 ss. no 4), glass, and ceramic surfaces of 26×76 mm in area were fabricated. These surfaces were rinsed with distilled water, dried, and then autoclaved at $121^{o}C$ for 15 min.

Food samples

Beef and lettuce samples were obtained randomly from a market in the Cheongju area. Beef and lettuce samples were cut to 26×76 mm in area and 1.0 mm thick. Each piece was put into a pre-sterilized Petri dish and artificially inoculated with FCV. The concentration of FCV was $1 \times 10^{6.75}$ $TCID_{50}$/mL. The beef was stored at $-20^{o}C$ and the lettuce was stored at $4^{o}C$ until use.

Treatments

Physical treatments included ultraviolet irradiation (wavelength, 270.0 nm), drying, freezing ($-20^{o}C$), and rinsing with tap water. Chemical treatments included sodium bicarbonate, hydrogen peroxide (Sigma-Aldrich, St. Louis, MO, USA), ethanol (Merck KGaA Co., Darmstadt, Germany), iodine (Shinyo Pure Chemical Co., Osaka, Japan), ammonium chloride (Tedia Co., Inc., Fairfield, OH, USA), isopropanol (Jin, Seoul, Korea), and trisodium phosphate (TSP; Showa Chemical Co., Tokyo, Japan). The organic acids used included acetic acid (Shinyo), benzoic acid (Sigma-Aldrich), citric acid (DC Chemical Co., Seoul, Korea), lactic acid (Tedia), and propionic acid (Sigma-Aldrich).

Virus and cells

FCV strain F9 (catalog no. VR-782) was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and propagated in Crandell-Reese feline kidney (CRFK) cells from the Korean Cell Line Bank (KCLB, Seoul, Korea). The cells were grown in Dulbecco's modified Eagle's medium (DMEM; Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone), 1% 10 mM non-essential amino acids (NEAA; Gibco, Grand Island, NY, USA), and 1% penicillin-streptomycin (Gibco). Cells were incubated at $37^{o}C$ in a humidified chamber under 5% CO_2, and then divided into separate culture dishes when they were over 90% proliferated (12,13,25). CRFK cell monolayers at 90% confluence were inoculated with FCV and incubated at $37^{o}C$ in the maintenance medium (DMEM, 5% FBS, 1% penicillin-streptomycin, and 1% NEAA) for $3 \sim 4$ days. Virus stocks were stored in cryogenic vials (Nalgene, Rochester, NY, USA) in liquid nitrogen. Viruses were thawed in a $37^{o}C$ water bath for 10 min prior to artificial inoculation.

Tissue culture infectious dose$_{50}$ ($TCID_{50}$)

CRFK cells were maintained as stock cultures in DMEM and re-plated 2 days before infection in 96-well plates for $TCID_{50}$ assays (11,14). Treated samples and their paired controls were subjected to 10-fold serial dilutions. The medium used for dilution and infection was Dulbecco's phosphate-buffered saline pH 7.4 (DPBS; Gibco). Samples of each dilution (20 µL) were deposited in each of 10 wells in a 96-well plate. Following a 1-hr incubation at $37^{o}C$ in a humidified chamber under 5% CO_2, 80 µL of maintenance medium was added to each well. After 4 days of incubation, the cells were observed for cytopathic effects. The plate was washed and the remaining cells were stained with 0.5% methylene blue in 50% ethanol. The 50% tissue culture infectious dose ($TCID_{50}$) was calculated according to the method of Reed and Muench (26).

Inactivation of viruses on food contact surfaces and food surfaces

The food contact surfaces examined here were stainless steel, glass, and ceramic. The food surfaces used were lettuce and beef. Food contact surfaces and food surfaces were inoculated with FCV suspensions (1 mL). To allow for absorption of FCV, the sample surfaces were incubated at $37^{o}C$ for 1 hr, and then physical, chemical, disinfectant, or organic acid treatments were administered. Sterile moistened cotton swabs were used to sample the surfaces by rubbing over an area of approximately 26×76 mm (27). The contaminated area was exposed to 20 µL of test solution or DPBS as a control. Treatment samples of each dilution (20 µL) were deposited in each of 10 wells in a 96-well plate. Samples from the swabs were diluted in DPBS pH 7.4. Twenty µL of the samples which dilutes was exposed in 96-well plate. After 80 µL of maintenance medium was added to a 96-well plate, the plate was agitated on a shaker platform at 150 rpm for 30 min. After 4 days of incubation, the cells were observed for cytopathic effects. Viral titers in the treated and control wells were compared to determine the extent of virus reduction.

Inactivation by physical treatment

The efficacy of four physical treatments was assessed. For the UV irradiation treatment, contaminated food contact surfaces and food surfaces were incubated under a germicidal lamp (length, 800 mm; wavelength, 270.0 nm; ozone free; DAE chung science, Daejeon, Korea) with a tubular glass envelope emitting short-wavelength UV radiation with a monochromatic peak at 270.0 nm for 10, 20, or 30 min. In the drying treatment, food contact surfaces or food surfaces were inoculated with FCV, allowed to dry for 10 min, 1, 3, 12, 24, or 48 hr, and then assessed. For the cold-storage treatment, surfaces were inoculated and then frozen at $-20^{o}C$ for 0.5, 1, 12, or 24 hr; the samples were then thawed and assessed.

For the tap water treatment, food contact surfaces were inoculated with FCV suspensions and immersed in tap water. Following 0.5, 1, 12, and 24 hr of treatment, the test samples were serially diluted and assayed immediately to determine the $TCID_{50}$.

Inactivation by chemical treatment

The following solutions were prepared in sterile distilled water: sodium bicarbonate (0.1, 0.5, 1, 5, 10, and 20%); ammonium chloride (0.1, 2, 6, and 10%); hydrogen peroxide (1, 10, 100, and 1000 ppm); iodine (5, 15, 25, and 35 ppm); isopropanol (40~60%); TSP (200, 1100, 3000, and 5900 ppm), and ethanol (60, 70, and 80%). The solubility of elemental iodine in water can be greatly increased by the addition of small amounts of chloroform or carbon tetrachloride, into which iodine easily dissolves. These solutions were evaluated for their inactivation of FCV dried onto food contact surfaces after an exposure of 1 min at room temperature; food contact surfaces exposed to 70% ethanol were also tested after a 30-sec exposure. The test suspensions were diluted serially and assayed immediately to determine the $TCID_{50}$.

Inactivation by organic acid treatment

Five different organic acids (acetic, benzoic, citric, lactic, and propionic acid) were tested on food surfaces (beef or lettuce) at concentrations of 100, 1000, 3000, or 6000 ppm. The test suspensions were diluted serially and assayed immediately to determine the $TCID_{50}$.

Calculation of disinfection efficacy

After each treatment, performance was analyzed in terms of the reduction in FCV titer. The efficacy evaluation was performed as described (20,28-30).

RESULTS AND DISCUSSION

Determination of $TCID_{50}$ for FCV

To measure the concentration of virus, the tissue cul-

ture infectious dose 50 ($TCID_{50}$) assay was applied. In this study, the concentration of FCV was $1 \times 10^{6.75}$ $TCID_{50}$/mL and serial dilutions of this suspension were used for each experiment.

FCV inactivation on food contact surfaces

Fig. 1 and Fig. 2 display the results for the various treatments used to disinfect FCV-contaminated surfaces. A treatment was considered effective if it caused a ≥ 4 $\log_{10}$ (99.99%) reduction in the number of infectious viral units compared to the untreated control. Generally, for antiviral efficacy testing, a 3 to 4 $\log_{10}$ reduction in virus titer compared with control is used (31,32). So, in this result, most treatments were able to cause 4 $\log_{10}$ reductions in FCV titer.

FCV was used in the present study as a model for NV inactivation by UV light (5,13,22,33). According to study, at least 99.99% inactivation would occur for FCV at the NSF-recommended dose of 40 mJ/cm^2 (33). Regarding the effect of UV irradiation on food contact surfaces, treatment for 30 min resulted in decreases of 99.42% and 99.41% on glass and ceramic surfaces, respectively (Fig. 1, A-3). A recent study reported that drying is relatively effective because virus on a metal surface would be directly exposed to dry air (6,29). We found that a drying time of 48 hr was most effective against FCV on food contact surfaces and that shorter exposure times were less effective. On stainless steel, freezing for 24 hr was most effective, with a 96.9% reduction (Fig. 1, B-4). This results show that freezing has limited effects in removing FCV. In contrast, freezing did not significantly reduce the viability of any of the viruses except the infectivity of FCV. The exceptions were FCV on food, in which the $TCID_{50}$ values were reduced by less than one $\log_{10}$ (5,34).

It has been demonstrated that transfer of the infectious viruses can readily occur by casual contact between
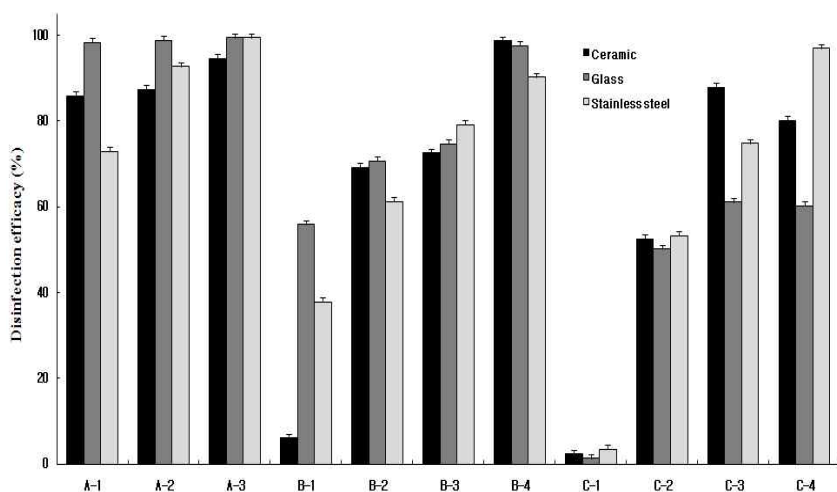
Fig 1. Comparison of the FCV inactivation efficacies of various physical treatments after inoculation of ceramic, glass, and stainless steel food contact surfaces. (A-1) UV 10 min; (A-2) UV 20 min; (A-3) UV 30 min; (B-1) drying 10 min; (B-2) drying 1 hr; (B-3) drying 12 hr; (B-4) drying 48 hr; (C-1) freezing 30 min; (C-2) freezing 1 hr; (C-3) freezing 12 hr; (C-4) freezing 24 hr.
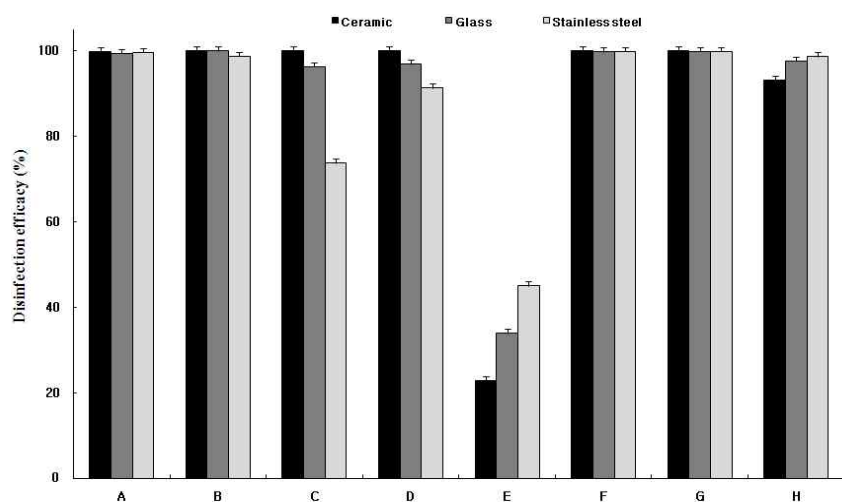
Fig 2. Comparison of the FCV inactivation efficacies of various chemical treatments (sodium bicarbonate, ammonium chloride, hydrogen peroxide, iodine, ethanol, isopropanol, and trisodium phosphate [TSP]) after inoculation of ceramic, glass, and stainless steel food contact surfaces. (A) sodium bicarbonate 5%; (B) ammonium chloride 10%; (C) hydrogen peroxide 100 ppm; (D) hydrogen peroxide 1000 ppm; (E) iodine 25 ppm; (F) ethanol 60%; (G) isopropanol 60%; (H) TSP 5900 ppm.

foods and environmental surfaces and that NV is relatively resistant to inactivation by chemical and physical agents. Effective disinfection of surfaces may be useful in preventing or reducing the spread of NV. Because of the toxicity associated with chemical disinfectants, many commercially available disinfectants cannot be directly applied to the food contact surfaces. The disinfection efficacies of various concentrations of sodium bicarbonate, ammonium chloride, hydrogen peroxide, iodine, ethanol, isopropanol, and TSP against FCV after exposures of 1 min are shown in Fig. 2. In the past, sodium bicarbonate has been shown to be effective against bacteria, fungi, and FCV (12,25,28,35). Sodium bicarbonate at concentrations of 0.1~20% reduced 86.2~99.73% of FCV (data not shown), with the most powerful effect at 5% sodium bicarbonate, which killed 99.76, 99.38, and 99.57% of the virus on ceramic, glass, and stainless steel surfaces, respectively (Fig. 2, A). Quaternary ammonium-based compounds are reported to be the least effective against hydrophilic, non-enveloped viruses, such as FCV, canine parvovirus, and poliovirus (17,36). In one study, quaternary ammonium-based compounds alone to be ineffective, but in combination with sodium bicarbonate they showed some anti-FCV activity. In these experiments, quaternary ammonium-based compounds were used in combination with sodium bicarbonate, and were able to cause a more than 3 $\log_{10}$ reduction in FCV titer on stainless steel surfaces (12,19). Ammonium chloride concentrations of 0.1~10% appeared to be more effective, with 99.9% virus inactivation occurring within 1 min (data not shown). The weakest inactivation was observed with 10% ammonium chloride, which inactivated 98.71% of virus on stainless steel (Fig. 2, B). Hydrogen peroxide has been reported to be a potent disinfectant (36-38); however, higher concentrations of hydrogen peroxide sometimes lead to browning of produce

(36). On the ceramic surface, a hydrogen peroxide concentration of 1 ppm resulted in 99.99% inactivation, and the most potent effect was obtained with 100 ppm hydrogen peroxide, which killed 99.98, 99, and 99.25% of the virus on ceramic, glass, and stainless steel surfaces, respectively (Fig. 2, C-D). In previous studies, iodine was relatively ineffective, even at 300 ppm (12,17); this is a concern, considering that a maximum of 75 ppm is recommended by manufacturers for environmental surface disinfection, and the Korean Food and Drug Administration recommends 25 ppm for sanitizing food contact surfaces (9). In this study, lower concentrations of iodine (5~35 ppm) exhibited less than 50% disinfection efficacy (data not shown). Within the iodine treatments, 25 ppm iodine provided the best results, killing 22.91, 33.94, and 45.05% of the virus on ceramic, glass, and stainless steel surfaces, respectively (Fig. 2, E). In general, the most effective ethanol concentrations reported are greater than 50%. However, in a previous study, ethanol concentrations greater than 70% were ineffective against FCV after a 1-min exposure (21). In the present study, ethanol was slightly less effective than isopropanol. This may be due to the reduced hydrophilic nature of FCV, which makes the virus more susceptible to isopropanol. In a previous study using cell suspensions, they found that isopropanol at 50~70% was more effective than 50~80% ethanol, killing more than 99.99% of FCV in less than 1 min (20). In this study, ethanol concentrations of 60, 70, and 80% appeared to be more effective than isopropanol, with 99.99% virus inactivation occurring within 1 min (data not shown). Among isopropanol treatment, a concentration of 60% had the most potent effect on FCV titers on food contact surfaces, killing 99.93, 99.83, and 99.88% of the virus on ceramic, glass, and stainless steel surfaces, respectively (Fig. 2, G). TSP was used alone or in the presence of

nisin, since these treatments have been previously shown to be effective against other pathogens in culture and in foods. For example, 10 min with 10% TSP at 48°C, followed by incubation in the presence of 500 IUmL^{-1} nisin, resulted in no viable cells being recovered after 24 hr (2 log$_{10}$ reduction cell kill) indicating that a multiple hurdle approach is the most effective method of reducing growth and survival of *A. butzleri* in culture (39). TSP at 200~5900 ppm killed 1.62~98.59% of FCV (data not shown); the greatest inactivation was observed at 5900 ppm, which killed 93.08, 97.6, and 98.59% on ceramic, glass and stainless steel surfaces, respectively (Fig. 2, H).

Disinfection of FCV on food surfaces

The results of the physical treatments are shown in Fig. 3. To evaluate the effect of UV irradiation on contaminated beef or lettuce, inoculated food samples were irradiated for 10, 20, or 30 min. The 30-min UV treatment provided the best results for FCV on beef (99%) and lettuce (99.9%). Similarly, air-drying for 48 hr inactivated 99.92 and 99.97% of the virus found on beef and lettuce, respectively (Fig. 3, B-4). We found that a drying time of 48 hr was most effective against FCV on food contact surfaces and that shorter exposure times were less effective. Similar to the UV treatment results, inactivation by drying was slightly more effective for lettuce than beef.

The disinfection efficacy of various concentrations of acetic acid, benzoic acid, citric acid, lactic acid, and propionic acid against FCV after 1 min of contact is shown in Fig. 4. FCV-inoculated samples were treated with 100, 1000, 3000, or 5900 ppm of each acid. Of the organic acids tested, propionic acid was most effective. Acetic acid and propionic acid at 3000 ppm were most effective, whereas benzoic acid, citric acid and lactic acid were more effective at 5900 ppm. Acetic acid at concen-

trations of 100 to 5900 ppm concentration killed 87.98 to 99.84% of FCV. The most potent effect was observed with a concentration of 5900 ppm, which killed 99.15 and 99.84% of the virus in beef and lettuce, respectively (Fig. 4, B). Benzoic acid at concentrations of 100 to 5900 ppm killed 97.49 to 99.93% of FCV. The potent effect was observed with a concentration of 3000 ppm, which killed 99.92 and 99.93% of the virus in beef and lettuce, respectively (Fig. 4, C). Citric acid and lactic acid showed similar results compared to benzoic acid. At a concentration of 3000 ppm, citric acid killed 99.45 and 99.81% of the virus in beef and lettuce, respectively (Fig. 4, D). On beef and lettuce, lactic acid at a concentration of 3000 ppm was found to be the most effective, killing 99.57% and 99.26% of the virus, respectively (Fig. 4, E). Propionic acid concentrations of 1000 ppm appeared to be more effective than the other organic acids, with 99% virus inactivation occurring within 1 min (data not shown). Propionic acid was most effective at a concentration of 5900 ppm, killing 99.88% and 99.99% of the virus on beef and lettuce, respectively (Fig. 4, G). Overall, most organic acid treatments showed a disinfection efficacy >99%. These results are consistent with previous studies showing that FCV is quite unstable at lower pH values. However, it is important to note that most entero viruses are generally acid resistant, which is probably crucial for surviving the stomach environment and reaching the target cells in the small intestine (40). For example, the stability of FCV in a wide pH range, i.e., less than complete inactivation for pH 6, was clearly higher than the pH-dependent stability of CaCV. With respect to the pH stability of the enteric noroviruses, it was shown that 3 hr at pH 2.7 (at room temperature) was not enough to completely inactivate NV in gastrointestinal conditions (i.e., low pH and high bile concentrations), other enteric viruses (e.g.,
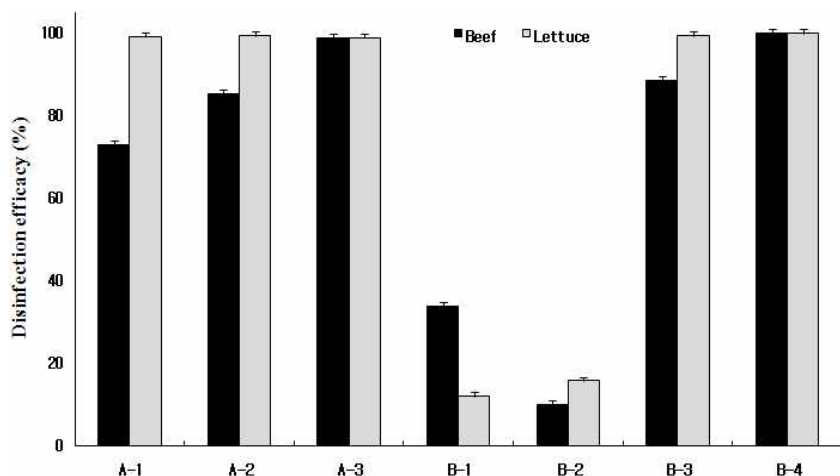
Fig 3. Comparison of the FCV inactivation efficacies of UV irradiation and drying after the inoculation of food surfaces (beef or lettuce). (A-1) UV 10 min; (A-2) UV 20 min; (A-3) UV 30 min; (B-1) drying 10 min; (B-2) drying 1 hr; (B-3) drying 12 hr; (B-4) drying 48 hr.
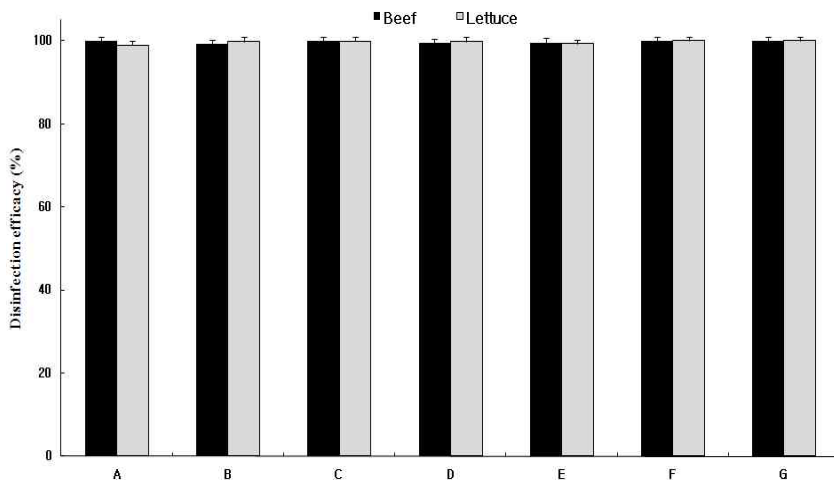
Fig 4. Comparison of the viral inactivation efficacies of organic acids in FCV-inoculated food surfaces (beef or lettuce). (A) acetic acid 3000 ppm; (B) acetic acid 5900 ppm; (C) benzoic acid 3000 ppm; (D) citric acid 3000 ppm; (E) lactic acid 3000 ppm; (F) propionic acid 3000 ppm; (G) propionic acid 5900 ppm.

poliovirus, hepatitis A virus, or rotavirus) might be better than the animal caliciviruses (13). Furthermore, organic solvent extraction is often an essential step in concentrating enteric viruses from environmental, food, and water samples (6,22,35,36,38).

Comprehensive analysis of the various treatments

NVs are very stable in the environment (21). Therefore, in addition to direct person-to-person transmission, they may also be transmitted via contaminated environmental surfaces and hands. Thus, food handlers with contaminated hands represent a serious problem, as they may cause large-scale outbreaks. For this reason, the treatment of environmental surfaces and hands is very important to prevent NV transmission. The use of surrogate cultivable viruses has been recognized by the Environmental Protection Agency for the testing of antiviral disinfectants. The closely related FCV has been used previously as a model for NV inactivation studies (16). Considering the physicochemical and structural similarities between NV and FCV, it is reasonable to assume that an agent demonstrated to kill FCV will also kill NV when used under identical conditions. In a recent study, most commercial disinfectants proved to be ineffective against FCV at the manufacturer's recommended concentrations (12). In this study, the FCV-inactivating efficacy of various agents was quantified using inoculated ceramic, glass, and stainless steel surfaces. Regarding food contact surfaces, most agents proved to be more effective on ceramic or glass than on stainless steel. On ceramic and glass, FCV inactivation was highly effective with the use of chemical treatments and organic acids. However, iodine demonstrated a very low FCV inactivation efficacy on food contact surfaces. Regarding food surfaces, most agents proved to be more effective on lettuce than on beef. This suggests that the difference in surface texture of lettuce and beef may affect the virus

survival. Organic acids may have been inactivated due to interactions with proteins on the beef surface. However, organic acid treatments were very effective overall on food surfaces. On food contact surfaces, ceramic surfaces were most easily disinfected, whereas stainless steel surfaces were more difficult to disinfect. Overall, the chemical treatments were more effective than the physical treatments for both food contact surfaces and food surfaces, perhaps because chemical treatments cause denaturation of viral capsid proteins.

In this study, we confirmed the effectiveness of commonly used sanitizers against the propagation of FCV, a virus very similar to that causing viral gastroenteritis in humans. Thus, these findings could be applied to prevent the cross-contamination of foodborne viruses and the subsequent spread of NV-associated foodborne illnesses.

ACKNOWLEDGEMENT

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