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Efficacy of Neutral Electrolyzed Water for Inactivation of Human Norovirus

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ABSTRACT Human norovirus (NoV) is the leading cause of acute gastroenteritis worldwide. Persistence on surfaces and resistance to many conventional disinfectants contribute to widespread transmission of norovirus. We examined the efficacy of neutral electrolyzed water (NEW; pH 7) for inactivation of human NoV GII.4 Sydney in suspension (ASTM method 1052-11) and on stainless steel surfaces (ASTM method 1053-11) with and without an additional soil load. The impact of the disinfectant on viral capsid was assessed using reverse transcriptase quantitative PCR (RT-qPCR; with an RNase pretreatment), SDS-PAGE, transmission electron microscopy, and a histo-blood group antigen (HBGA) receptor-binding assay. These studies were done in parallel with those using Tulane virus (TuV), a cultivable human NoV surrogate. Neutral electrolyzed water at 250 ppm free available chlorine produced a 4.8- and 0.4-$\log_{10}$ reduction in NoV genome copy number after 1 min in suspension and on stainless steel, respectively. Increasing the contact time on surfaces to 5, 10, 15, and 30 min reduced human NoV genomic copies by 0.5, 1.6, 2.4, and 5.0 $\log_{10}$ and TuV infectious titers by 2.4, 3.0, 3.8, and 4.1 $\log_{10}$ PFU, respectively. Increased soil load effectively eliminated antiviral efficacy regardless of testing method and virus. Exposure to NEW induced a near complete loss of receptor binding (5 ppm, 30 s), degradation of VP1 major capsid protein (250 ppm, 5 min), and increased virus particle aggregation (150 ppm, 30 min). Neutral electrolyzed water at 250 ppm shows promise as an antinoroviral disinfectant when used on precleaned stainless steel surfaces.

IMPORTANCE Norovirus is the leading cause of acute viral gastroenteritis worldwide. Transmission occurs by fecal-oral or vomitus-oral routes. The persistence of norovirus on contaminated environmental surfaces exacerbates its spread, as does its resistance to many conventional disinfectants. The purpose of this research was to evaluate the antinoroviral efficacy of neutral electrolyzed water (NEW), a novel chlorine-based disinfectant that can be used at reduced concentrations, making it more environmentally friendly and less corrosive than bleach. An industrial-scale electrochemical activation device capable of producing relatively stable electrolyzed water at a wide pH range was used in this study. Experiments showed that 250 ppm NEW effectively eliminated (defined as a 5-$\log_{10}$ reduction) human norovirus GII.4 Sydney (epidemic strain) on clean stainless steel surfaces after a 30-min exposure. Supporting studies showed that, like bleach, NEW causes inactivation by disrupting the virus capsid. This product shows promise as a bleach alternative with antinoroviral efficacy.

KEYWORDS disinfection, environmental contamination, norovirus, public health, surrogate, virus inactivation

Received 20 March 2017 **Accepted** 6 June 2017

Accepted manuscript posted online 9 June 2017

Citation Moorman E, Montazeri N, Jaykus L-A. 2017. Efficacy of neutral electrolyzed water for inactivation of human norovirus. Appl Environ Microbiol 83:e00653-17. https://doi.org/10.1128/AEM.00653-17.

Editor Donald W. Schaffner, Rutgers, The State University of New Jersey

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Human norovirus (NoV) is the leading cause of acute viral gastroenteritis worldwide (1). Despite its prominence as a foodborne pathogen (2), the majority of disease burden is caused by direct contact with infected individuals and their gastrointestinal

bodily fluids (feces, vomit). Both vomiting and fecal incidents lead to contamination of environmental surfaces and contribute to virus transmission (3, 4). Previous works demonstrating the shedding of virus at high concentrations by infected individuals (5) and long-term environmental persistence (3, 6–8) support the importance of environmental surfaces as reservoirs for human NoV transmission.

Hand hygiene and environmental sanitation practices, the latter of which utilize surface disinfectants and sanitizers, are employed to prevent and control the spread of human NoV in many different settings, for instance in health care and long-term care facilities, schools, and cruise ships (9). The current U.S. CDC recommendation for disinfecting solid surfaces presumably contaminated with human NoV is sodium hypochlorite (household bleach) at 1,000 to 5,000 ppm free chlorine, depending upon the circumstances of the contamination event (9). Although bleach at these concentrations has been shown to reduce levels of human NoV by >4 $\log_{10}$ reverse transcriptase quantitative PCR (RT-qPCR) units in suspension assays (6), perpetual implementation of this recommendation may not be practical due to the corrosive nature of chlorine (at >500 ppm) and potential health hazards (10). Conversely, active ingredients within several classes of conventionally used surface disinfectants, e.g., quaternary ammonium compounds (QACs) and ethanol, do not fully inactivate human NoV at manufacturer recommended concentrations and contact times (6, 8, 11). Residual infectious virus on surfaces resulting from incomplete chemical inactivation has significant public health implications, primarily by prolonging the duration and magnitude of outbreaks.

Electrolyzed water, also known as electrochemically activated solution, is one class of emerging disinfectants that functions as a broad-spectrum, aqueous chemical oxidant generated by passing a dilute sodium chlorine solution through an electrolytic cell in a process known as electrolysis. Electrolyzed water has been evaluated for several food safety industrial applications, including use as a fresh produce wash (12) as well as for cleaning and disinfection in dairy manufacturing clean-in-place systems (13). The antimicrobial efficacy of electrolyzed water is attributed to pH, chlorine content (balance of chlorine gas [Cl_2], hypochlorous acid [HOCl], and hypochlorite ion [OCl^-]), and oxidation-reduction potential (ORP). HOCl is a more effective biocide relative to its dissociated form (OCl^-), which is the active ingredient of bleach. Manipulating the pH-dependent aqueous chemistry of electrolyzed water to a near neutral pH ensures that the $HOCl^-$ molecule predominates (14). Neutral electrolyzed water (NEW; pH 7) has been shown to be effective at reducing or eliminating bacterial pathogens (14) and cultivable human NoV surrogates, e.g., murine norovirus (15, 16). However, its ability to inactivate human NoV has not been established.

The purpose of this study was to examine the efficacy of NEW for inactivation of human NoV. In the absence of a readily available infectivity assay for human NoV and in order to amass a body of evidence supporting or refuting the efficacy of NEW, we used a multipronged approach. Specifically, we characterized the effect of NEW on human NoV viral genome and capsid integrity using a combination of RT-qPCR, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transmission electron microscopy (TEM), and a receptor-binding assay. Additionally, we evaluated the performance of the product using the most recent cultivable surrogate, Tulane virus (TuV), under the same experimental conditions.

RESULTS

NEW stability. The free available chlorine (FAC), ORP, and pH values of freshly prepared NEW were 270 ppm, 939.7 mV, and 6.50, respectively. Physiochemical properties of NEW remained relatively stable over a 7-day period, retaining FAC, ORP, and pH average values of 250 $\pm$ 2.0 ppm, 974.7 $\pm$ 2.7 mV, and 6.2 $\pm$ 0.0, respectively, with negligible fluctuations over time ($P > 0.05$). The antinoroviral activity of the solution against human NoV GII.4 Sydney in suspension assays did not indicate any significant change over storage time ($P > 0.05$, data not shown).

Efficacy of NEW based on RT-qPCR analysis. Neutral electrolyzed water was screened for antinoroviral efficacy using the following two American Society for Testing

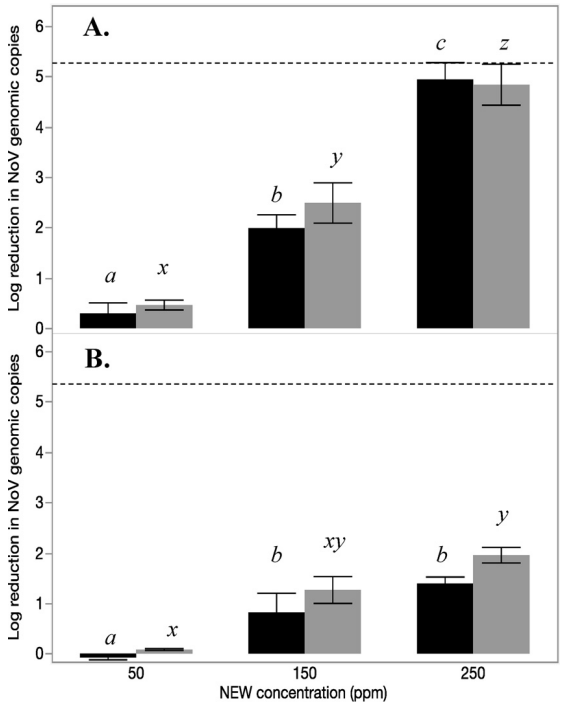


FIG 1 Inactivation of human NoV without (A) and with (B) additional soil load following a 1-min exposure to neutral electrolyzed water (NEW) in suspension assay followed by RT-qPCR. Black and gray bars indicate samples with and without an RNase pretreatment prior to RNA extraction and amplification, respectively. Experiments were performed in triplicate, and error bars indicate standard error. Different letters show statistically significant differences ($P < 0.05$) when comparing $\log_{10}$ reduction in genomic copies by concentration of NEW for samples without (*a*, *b*, *c*) and with (*x*, *y*, *z*) RNase pretreatment. The dashed lines indicate the highest quantifiable degree of virus $\log_{10}$ inactivation based on assay limit of detection.

and Materials (ASTM)-approved methods with minor modification: suspension assay (E1052-11) (17) and surface test (E1053-11) (18), both with and without added soil load. Human NoV inactivation following a 1-min exposure to 50, 150, and 250 ppm FAC in suspension is shown in Fig. 1. Only the 250-ppm NEW concentration, in the absence of supplemented soil load, produced greater than a 5.4-$\log_{10}$ reduction in NoV genome copy number. There were no statistically significant differences at any one concentration between samples processed with or without RNase pretreatment. Statistically significant differences ($P < 0.05$) were observed when comparing the three NEW concentrations to each other as well as when comparing samples with and without added soil load treated at any given NEW concentration.

Stainless steel coupons were used to evaluate the antinoroviral efficacy of NEW on nonporous surfaces. Because of the superior efficacy of the 250-ppm FAC treatment, this concentration was chosen for the surface studies, with contact times ranging from 1 to 30 min (Fig. 2). At 250 ppm FAC, NEW produced a 1.6 ± 0.7, 2.4 ± 0.5, and 5.0 ± 0.5 $\log_{10}$ reduction in genomic copies after 10, 15, and 30 min, respectively ($P < 0.05$), when experiments were done without additional soil load. Soil load significantly reduced NEW efficacy ($P < 0.05$), producing a $<$0.3-$\log_{10}$ genomic copy number reduction following exposure regardless of contact time and RNase treatment ($P > 0.05$).

Evidence of viral capsid degradation and loss of receptor binding upon exposure to NEW. SDS-PAGE was performed to provide supportive evidence of human NoV capsid degradation following exposure to NEW. As shown in Fig. 3, untreated human NoV virus-like particles (VLPs) produced two bands corresponding to the VP1 and cleaved VP1 (cVP1) proteins, with respective molecular masses of approximately 50 kDa and 60 kDa. Exposure to lower concentrations of NEW resulted in minimal reductions

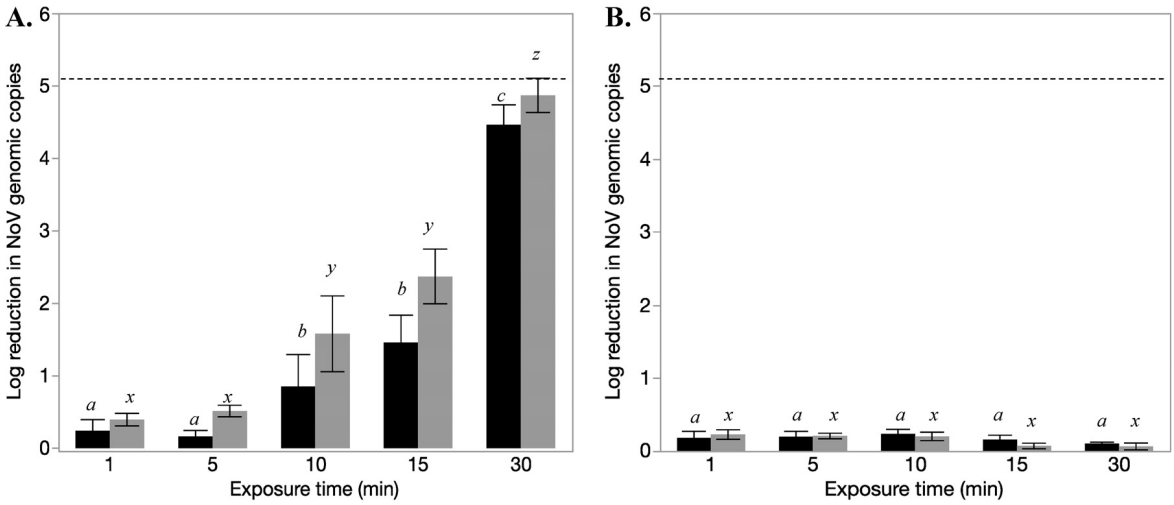
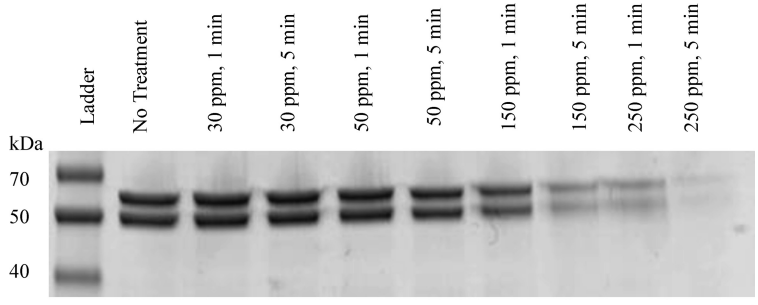


FIG 2 Inactivation of human NoV on stainless steel coupons following exposure to NEW. Viral inoculum without (A) and with (B) additional soil load was dried onto stainless steel coupons and treated with NEW (250 ppm, pH 7.0). Samples were analyzed by RT-qPCR without (black bars) and with (gray bars) RNase pretreatment. Experiments were performed in triplicate, and error bars indicate standard error. Different letters show statistically significant differences ($P < 0.05$) when comparing $\log_{10}$ reduction in genomic copies by concentration of NEW for samples without (*a*, *b*, *c*) and with (*x*, *y*, *z*) RNase pretreatment. The dashed lines indicate the highest quantifiable degree of virus $\log_{10}$ inactivation based on assay limit of detection.

in protein band intensities (reductions of <12%, 9%, and 35% following treatment with 30, 50, and 150 ppm FAC for 1 min, respectively). Treatment at a higher concentration (250 ppm FAC) and longer contact time (5 min) produced a 97% reduction in band intensity. Transmission electron microscopy images showed loss of capsid structural integrity and increased VLP aggregation following exposure at 150 ppm FAC for 30 min (Fig. 4) but not after 10 min (data not shown), providing further support for capsid degradation upon exposure to this disinfectant.

Receptor-binding assays were used as an indirect measurement of virus infectivity. A_{450} measurements ranged from a low of 0.15 (nonnormalized negative control) to 2.0 (positive control). The ability of human NoV VLPs to bind histo-blood group antigen (HBGA) receptors was quantified following treatment with NEW for 30 s. Receptor binding was significantly reduced by 31.9% ± 15.7% and 51.1% ± 2.0% ($P < 0.05$) following treatment with 5 and 10 ppm FAC, respectively. Near complete abolishment of receptor binding (>98%) was achieved following exposure to 15 and 20 ppm FAC.

Impact of NEW on Tulane virus surrogate infectivity. When solid stainless steel coupons were inoculated with ~7.0 $\log_{10}$ TuV cell culture lysate, dried, and treated with



Reduction in band intensity (%)	0	11.3	16.7	8.8	18.6	34.8	81.9	66.6	97.4

FIG 3 SDS-PAGE analysis of human NoV VLP major capsid protein. Two-microliter aliquots of purified human NoV GII.4 Grimsby VLPs were dried onto stainless steel coupons and treated with neutral electrolyzed water at various concentrations and exposure times.

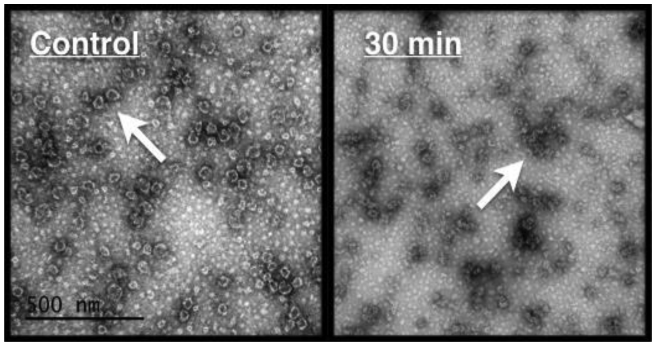


FIG 4 Impact of neutral electrolyzed water (150 ppm FAC, 30 min) on human NoV VLP capsid integrity by transmission electron microscopy (magnification, ×75,000). White arrows in the left and right images indicate intact (positive control) and aggregated virus particles (after treatment with NEW), respectively.

250 ppm FAC, infectivity reductions of 3.0 ± 0.5, 3.8 ± 1.0, and 4.1 ± 0.7 $\log_{10}$ PFU were observed after 10, 15 and 30 min, respectively (Fig. 5). In elevated soil load conditions, NEW (250 ppm FAC) was less effective against TuV, providing infectivity reductions of 1.0 ± 0.1, 1.0 ± 0.2, and 1.2 ± 0.1 $\log_{10}$ PFU after 10, 15, and 30 min, respectively. These data were significantly lower than those observed for assays without added soil load ($P < 0.05$).

DISCUSSION

Human NoV is one of the most prevalent pathogens worldwide, and evidence continues to mount that contaminated environmental surfaces enhance its transmission and intensify the duration and magnitude of outbreaks (3, 7). Unfortunately, many commonly used surface disinfectants, e.g., ethanol and quaternary ammonium compounds (QACs), do not completely inactivate human NoV at manufacturer recommended concentrations and contact times (6, 8, 11). Although chlorine bleach at 1,000 to 5,000 ppm FAC is the current CDC recommendation for human NoV inactivation on solid surfaces (9), this is an unrealistically high concentration for routine use in commercial and institutional settings. There is a need for more effective surface disinfec-

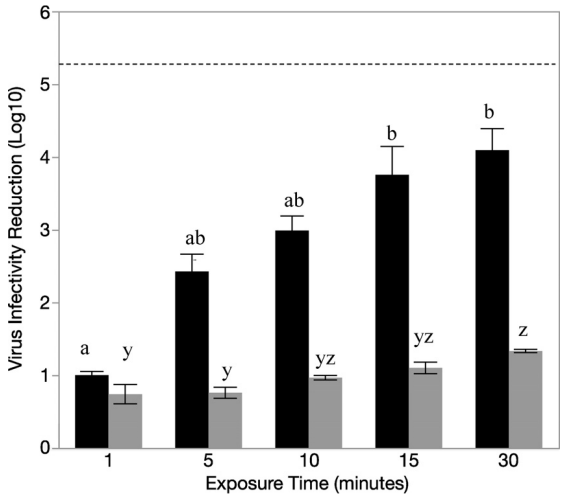


FIG 5 Inactivation of Tulane virus on stainless steel following exposure to neutral electrolyzed water (250 ppm FAC). Viral inoculum without (black bars) and with (gray bars) additional organic load was dried onto stainless steel coupons and treated with NEW (250 ppm FAC). Samples were analyzed for infectivity by plaque assay. Experiments were repeated in triplicate, and error bars indicate standard error. Letters above bars indicate statistically significant differences ($P < 0.05$) when comparing $\log_{10}$ reduction in infectivity to corresponding exposure times within the same treatment (without [a,b] or with [y, z]) soil load. The dashed line indicates the highest quantifiable degree of virus $\log_{10}$ inactivation based on the assay limit of detection.

tants having enhanced efficacy against human NoV but with reduced corrosive properties and better safety profiles. The purpose of this study was to examine one of these products, neutral electrolyzed water, for its antinoroviral efficacy.

Electrolyzed water technology has gained increasing interest due to the ability to generate potent oxidative disinfectants inexpensively and with low environmental impact. The technology is based on electrolysis or the use of a direct electric current to drive a chemical reaction that would otherwise not occur. In the case of NEW, electrolysis of sodium chloride in water results in the pH-dependent production of chlorine oxidants, such as chlorine gas, hypochlorous acid, and hypochlorite ion (14). Electrolyzed water in its acidic, neutral, and alkaline forms has been shown to have antimicrobial effects against foodborne and hospital-acquired bacterial, viral, and parasitic pathogens. Electrolyzed water solutions with pH values between 2 and 7 contain primarily HOCl, a potentially advantageous feature due to the increased antimicrobial activity of this molecule relative to hypochlorite ion, the active ingredient in chlorine bleach. Further, due to its neutral properties, NEW may be less corrosive than acidic or alkaline disinfectants to contact surfaces particularly at extended contact times (10, 14), although this was not the focus of our research. Of course, as is the case for any disinfectant, concentration and contact time have a significant impact on efficacy.

In the absence of a simple and reliable *in vitro* cultivation method for human NoV, it is necessary to rely on culture-independent methods, more specifically RT-qPCR, for virus detection and quantification. An important limitation of nucleic acid amplification is its inability to differentiate between infectious and noninfectious virus particles, frequently underestimating the efficacy of a given inactivation strategy. There are a number of methodological alterations that have been proposed to ameliorate this problem, one of the more popular being RNase pretreatment (19). We employed this method and in so doing demonstrated both concentration and time-related differences in disinfection efficacy associated with exposure of human NoV to NEW. In suspension using no additional soil load, NEW at 250 ppm FAC was effective against the epidemic human NoV GII.4 Sydney strain, producing a 4.8-$\log_{10}$ reduction in RNA copy number. Park et al. (15) previously showed high efficacy for this product, using a GII.4 strain in a 1% fecal suspension as inoculum and endpoint titration RT-qPCR for quantification of product efficacy.

Compared to suspension tests, the ability of NEW to inactivate human NoV on stainless steel surfaces (measured by RT-qPCR both with and without RNase treatment) at the same concentration and exposure time (250 ppm FAC, 1 min) was significantly reduced. Such differences in disinfection efficacy between suspension and surface assays have been reported for other products and other microorganisms (16, 20). In addition to being suspended in a complex matrix and present in aggregated form (20–22), desiccation on surfaces may reduce physical access of the disinfectant to the virus particles (15). Samandoulgou et al. (23) also showed that hydrophobic and van der Waals interactions, as well as isoelectric point and ionic strength, may promote adhesion of nonenveloped viruses to solid surfaces, also decreasing access of the disinfectant to the virus. Our results support the mounting body of evidence suggesting that surface assays are a more conservative estimate of antiviral efficacy and should serve as the basis for evaluating disinfection efficacy for use in real-world, practical applications.

The efficacy of NEW for surface disinfection improved with increasing contact time, such that a 250-ppm FAC exposure for 15 to 30 min produced a greater than 3-$\log_{10}$ reduction in human NoV genomic copies. From a practical standpoint, this prolonged contact time may not be reasonable for environmental disinfection practices. Conversely, disinfection practices typically require a precleaning step to remove organic matter and dislodge microbes from surfaces, meaning that shorter contact times may be feasible. It should be noted that we did not include a physical force or removal step in our experiments, and future studies should focus on inclusion of this step. With

physical removal, a higher disinfection efficacy with perhaps shorter contact times may be obtained.

Consistent with the increased resistance of murine norovirus to chemical disinfection in the presence of elevated tryptone concentrations (16), we also observed that the efficacy of NEW against human NoV and TuV was negatively impacted by soil load. During redox reactions between chlorinated compounds and organic matter, reactive free available chlorine species, primarily HOCl (at neutral pH), convert to less reactive chemicals, including mono- and dichloramines as well as *N*-chloro compounds (24). It is well established that at identical chlorine concentrations, free chlorine has a much higher biocidal efficacy relative to that of its combined form (14).

As discussed above, the difficulty of reliably discriminating between infectious and noninfectious virus using RT-qPCR, even with an RNase pretreatment, remains an impediment in evaluating disinfectant efficacy for human NoV (19). For this reason, we characterized the effect of NEW on virus capsid integrity using other complementary methods, i.e., SDS-PAGE, TEM, and receptor-binding assay. SDS-PAGE analysis showed almost complete loss of the VP1 protein signal following exposure of VLPs to NEW at 250 ppm for 5 min, with TEM supporting significant capsid degradation after 10 min at 150 ppm FAC. Nowak et al. (25) suggested that capsid damage/destruction is the primary mode of chlorine-mediated viral inactivation. This is consistent with other studies (26, 27). Our results demonstrating human NoV inactivation through degradation of the viral capsid (TEM and SDS-PAGE analyses) are aligned with the aforementioned findings, as well as with those of Sano et al. (28) who quantified the formation of oxidatively produced carbonyl groups upon exposure to free chlorine as a proxy for human NoV capsid integrity and overall viral infectivity.

Human NoV inactivation as measured by reduction in HBGA binding was achieved very rapidly and at much lower concentrations ($<$20 ppm free available chlorine) compared to those of the other capsid integrity assays (e.g., RT-qPCR with RNase pretreatment, SDS-PAGE, and TEM). One explanation for this observation may be the high purity of the VLPs used in receptor-binding assays, which of course would have a very low chlorine demand, relative to the clarified 20% human NoV inoculum used in RT-qPCR experiments. As stated above, both virus aggregation and association with organic matter confer protective effects on the virus when exposed to physical and chemical stresses (16, 22). Another explanation may be the resolution of the receptor-binding assay, which encompasses an only 1- to 2-$\log_{10}$ VLP concentration, much less than can be accommodated in a quantitative molecular assay.

Cultivable surrogate viruses (e.g., murine norovirus and feline calicivirus) are frequently used as proxies for human NoV in inactivation studies. However, for a given treatment, surrogates do not always perform identically nor do they accurately mimic the behavior of human NoV. The discrepancy between historic and current human NoV surrogates in response to physical and chemical inactivation measures was recently studied in a comprehensive manner (8). This group reported that TuV was the most resistant of all surrogates tested. Other advantages of TuV are greater genetic and phylogenetic relatedness to human NoV (29) and the capability to bind to A and B HBGAs, putative human NoV receptors (30).

At 250 ppm FAC, NEW produced 1.6-, 2.4-, and 5.0-$\log_{10}$ reductions in human NoV RNA copy number and 3.0-, 3.8-, and 4.1-$\log_{10}$ reductions in TuV infectious titer following 10-, 15-, and 30-min treatments, respectively, on stainless steel. A similar trend of increasing $\log_{10}$ inactivation as a function of exposure time to NEW (250 ppm FAC) was observed for both human NoV and TuV at all time points (Fig. 2 and 5). Overall, our data demonstrate comparable inactivation efficacy for human NoV (evaluated by RNase-RT-qPCR) and TuV (evaluated by infectivity assay), further supporting that NEW is an effective disinfectant against human NoV.

A mounting body of evidence continues to show that inactivating human NoV in food and environmental matrices is a challenge. Previous studies have shown that chlorine bleach, used in accordance with the CDC recommendation of 1,000 and 5,000 ppm on clean and dirty surfaces, respectively, is the most effective method for

inactivating human NoV on solid nonporous surfaces (31). In conclusion, the technology used in this study allows large-scale production of a stable, more environmentally friendly disinfectant capable of inactivating norovirus by destruction of viral capsid. Neutral electrolyzed water at 250 ppm FAC shows promise as an alternative antinoroviral surface disinfectant when used for 15 to 30 min under relatively clean conditions.

MATERIALS AND METHODS

Human NoV and virus-like particles. Stool specimens obtained from confirmed human NoV GII.4 Sydney outbreaks were provided by S. R. Green (North Carolina State Laboratory of Public Health, Raleigh, NC). These were diluted to 20% (wt/vol) by suspension in 1× phosphate-buffered saline (PBS) (pH 7.2), clarified by centrifugation at 3,100 × *g* for 2 min, and used as stock virus in experiments. This virus stock had a concentration of ~8 $\log_{10}$ genomic copies/ml. Fecal suspension drying experiments (dry weight/wet weight) indicated stocks contained 1.5% to 3.0% solids. Working fecal suspensions with additional soil load were prepared by adding 25 μl of 5% bovine serum albumin, 100 μl of 0.4% bovine mucin, and 35 μl of 5% yeast extract (wt/vol) into 340 μl of 20% fecal suspension according to the American Society for Testing and Materials (ASTM) (18). Additional soil load in the final inoculum was composed of 0.25% bovine serum albumin, 0.08% bovine mucin, and 0.35% yeast extract (wt/vol). GII.4 Grimsby and GII.4 New Orleans virus-like particles (VLPs) at respective concentrations of 1.4 and 1.1 μg/μl were provided courtesy of R. Atmar (Baylor College of Medicine, Houston, TX). The VLPs were prepared, purified, and their integrity was validated as previously reported (32). Fecal suspensions and VLPs were stored at −80°C and 4°C, respectively, until use.

Tulane virus stock preparation and enumeration. Tulane virus was propagated in rhesus monkey kidney (LLC-MK2) cells, both kindly provided by Xi Jiang (Cincinnati Children's Hospital Medical Center, Cincinnati, OH) using M199 medium (Corning, Manassas, VA) supplemented with 10% fetal bovine serum (FBS; Thermo Scientific, Waltham, MA) and 1% penicillin-streptomycin antibiotic (Thermo Scientific) as explained previously (29). For virus stock preparation, 90% confluent monolayers were infected with TuV and incubated at 37°C in 5% CO_2 for 48 h or until overt cytopathic effects were observed. Viruses were harvested by three consecutive rounds of freeze-thaw followed by centrifugation at 12,000 × *g* for 20 min, after which virus-laden supernatant was collected and stored at −80°C until use. Plaque assays for TuV quantification were done using LLC-MK2 cells as previously described (29). Virus titer, as determined by plaque assay on MK2 cells, was ~7 $\log_{10}$ PFU/ml.

Neutral electrolyzed water generation. Neutral electrolyzed water was generated using a Qlean-Genie model UL-75a device (Qlean Tech Enterprises, Mendota Heights, MN) according to the manufacturer's recommendations. Each batch was tested for concentration of free available chlorine (FAC) (the sum of the reactive chlorine species, i.e., HOCl, OCl^-, and Cl_2) using the iodometric titration method (Hach Co., Loveland, CO). The pH and oxidation-reduction potential (ORP) of each NEW batch were measured using a dual scale pH/ORP meter following proper calibration using the manufacturer's standard reagents (Orion Versa Star Pro; Fisher Scientific, Pittsburgh, PA). To reach the desired FAC concentration for testing purposes, the stock NEW solution was diluted with deionized (DI) water and used within 30 min of generation for all experiments. For subsequent stability testing purposes, 1 liter of freshly generated NEW solution was stored in a sealed container in a dark room at room temperature. NEW physiochemical properties, including pH, ORP, and FAC, were measured periodically over 7 days.

Disinfection protocols. Preliminary suspension assays were performed in accordance with ASTM method E1052-11, with minor modifications to account for reduced testing volumes (17). Briefly, 25 μl human NoV fecal suspension (with or without additional soil load) was suspended into 225 μl NEW at concentrations of 50, 150, and 250 ppm FAC (pH 7.0) for 1 min. Disinfectant activity was terminated by transferring 20 μl of virus/disinfectant solution into 180 μl of 10% D/E neutralization broth (Sigma-Aldrich, St. Louis, MO), and the sample was immediately extracted for RNA isolation as described below. As per the ASTM E1052-11 protocol (17), a positive control (25 μl fecal suspension added to 225 μl PBS) and a neutralization control (25 μl fecal suspension added to 225 μl neutralized disinfectant) were also performed.

Surface (carrier) tests were done in accordance with ASTM method E1053-11 (18) with minor modifications (i.e., lower sample volumes, stainless steel carriers instead of glass, and vortexing as a means to remove virus film instead of scraping). Nonadhesive stainless steel tape (Newell Rubbermaid, Atlanta, GA) was cut into 1.0 by 0.5 in. coupons, soaked in acetone for 2 min, and autoclaved (121°C, 15 min) prior to use. Coupons were placed in plastic disposable petri dishes, inoculated with 20 μl human NoV fecal stock or TuV cell culture lysate (with or without additional soil load), and allowed 2 h to dry in a biosafety cabinet. Then, 180 μl NEW (250 ppm FAC, pH 7.0) was pipetted onto coupons and held for 1, 5, 10, 15, and 30 min, after which the coupon and its entire liquid volume were transferred to a 15-ml conical tube containing 1.8 ml 10% D/E broth for neutralization. Virus elution from coupons was achieved by vortexing for 30 s.

RNA extraction and RT-qPCR. Because molecular amplification methods cannot be relied upon to definitively discern human NoV infectivity status, samples after disinfectant exposure received an RNase enzymatic treatment prior to RNA extraction (26). RNase degrades free viral RNA, the amplification of which may lead to an overestimation of the amount of infectious virus and subsequent underestimation of disinfectant efficacy. It was thus stipulated in this study that sample treatment with RNase prior to RNA extraction provides an indirect measurement of capsid dissociation, as only RNA from structurally intact

capsids will be detected by RT-qPCR. Alternatively, reductions in RNA obtained from RT-qPCR analysis without RNase pretreatment provided some insight into genome integrity after treatment (19). For RNase pretreatment, 2 μl RNase One and 22 μl reaction buffer (Promega, Madison, WI) were added to a 200-μl sample eluate and incubated at 37°C for 15 min. Samples were placed on ice for 5 min to abolish RNase enzyme activity prior to RNA extraction, which was done using an automated NucliSens easyMag system (bioMérieux, St. Louis, MO) in accordance with manufacturer recommendations. Extracted RNA was eluted in 25 μl proprietary buffer and stored at −80°C until analysis by RT-qPCR.

The conserved region at the ORF1-ORF2 junction of the human NoV GII genome was targeted in the amplifications. Primers JJV2F (5′-CAAGAGTCAATGTTTAGGTGGATGAG-3′) and COG2R (5′-TCGACGCCATC TTCATTCACA-3′) and probe RING2P (5′-FAM [6-carboxyfluorescein]-TGGGAGGGCGATCGCAATCT-BHQ-3′) were used as previously described (33). RT-qPCR was performed using the SuperScript III Platinum one-step quantitative RT-PCR system (Invitrogen, Carlsbad, CA) and a CFX96 Touch real-time PCR detection system (Bio-Rad, Hercules, CA). A 25-μl reaction mixture containing 7.55 μl water, 12.5 μl 2× reaction buffer, 0.5 μl of 10 μM forward and reverse primers, 0.5 μl of 10 μM TaqMan probe, 0.5 μl SuperScript III reverse transcriptase/Platinum *Taq* DNA polymerase, 0.25 μl RNasin Plus RNase inhibitor (Promega), and 2.5 μl RNA template was subject to the following amplification conditions: (i) reverse transcription for 15 min at 50°C; (ii) activation of Hot Start DNA polymerase for 2 min at 95°C; and (iii) 45 cycles of 15 s at 95°C, 30 s at 54°C, and 30 s at 72°C.

For purposes of standard curve generation, the *in vitro* MEGAshortscript high-yield transcription kit (Ambion, Austin, TX) was used to generate RNA transcripts at a concentration of 12 $\log_{10}$ RNA copies/μl as explained elsewhere (34). Briefly, viral RNA genome extracted from clinical human NoV GII.4 Sydney strains was amplified by reverse transcriptase PCR using T7-JJV2f/G2SKR primers. Amplified DNAs were extracted after gel electrophoresis and transcribed utilizing the MEGAshortscript transcription kit (Ambion, Austin, TX). The transcripts (~12 $\log_{10}$ genome copies/μl) were diluted in THE RNA storage solution (1 mM sodium citrate, pH 6.4; Ambion) and stored at −80°C until analysis.

Viral RNA quantification was performed by comparing RT-qPCR-derived cycle threshold (C_T) values of 10-fold serially diluted RNA transcripts to experimental samples. Virus inactivation was calculated by subtracting the $\log_{10}$-transformed genomic copy number of treatment samples from the genomic copy number corresponding to the neutralization control sample and reported as mean ± standard error. Virus inactivation for samples yielding nondetection via RT-qPCR analysis was calculated by subtracting the RT-qPCR assay limit of detection value (1.42 $\log_{10}$ genome copies) from the $\log_{10}$-transformed positive neutralization control values. This corresponds to an average maximum quantifiable degree of virus $\log_{10}$ inactivation of 5.4 $\log_{10}$ genome copies.

SDS-PAGE. SDS-PAGE experiments were performed using human NoV VLPs in accordance with ASTM method 1053-11 (18) with modified volumes. A 2-μl volume of GII.4 Grimsby VLPs (1.1 μg/μl) was added to stainless steel coupons and allowed to dry for 30 min. VLPs were then exposed to 5 μl NEW (30, 50, 150, and 250 ppm FAC) for 1 and 5 min. Reactions were terminated following the addition of 20 μl of 10% D/E neutralization broth. Sample eluates were mixed 1:1 with 2× Laemmli buffer (Bio-Rad, Hercules, CA) and heated for 5 min at 95°C. Electrophoresis was performed on 20-μl sample volumes at 200 V for 25 min using Mini-Protean TGX gels (Bio-Rad) and a Spectra multicolor broad-range protein ladder (Fisher Scientific, Fairlawn, NJ). Gels received a 30-min Coomassie blue stain (Bulldog Bio, Portsmouth, NH) and were destained by rinsing in 1× PBS (pH 7.2). The intensity of the bands was quantified using Image Studio software (LI-COR Biosciences, Lincoln, NE).

Transmission electron microscopy. Transmission electron microscopy imaging was performed on human NoV GII.4 Grimsby VLPs following suspension in NEW (150 ppm FAC, 30 min). The disinfectant-treated VLPs were added to carbon substrate grids (Ladd Research, Williston, VT) and negatively stained using 2% uranyl acetate. A JEOL 1210 transmission electron microscope (JEOL USA, Inc., Peabody, MA) located at the Center for Electron Microscopy (North Carolina State University, Raleigh, NC) was used for visualization set at 80 kV with a ×75,000 magnification.

Receptor-binding assay. A histo-blood group antigen (HBGA) receptor-binding assay was adapted from a previous study (35). VLP binding affinity for HBGAs was quantitated following a 30-s treatment with NEW (5, 10, 15, 20 ppm FAC). Medium-binding 96-well polystyrene plates (Costar 3591; Thermo Fisher Scientific) were coated with 0.2 μg of human NoV GII.4 New Orleans VLPs and incubated with gentle shaking at 4°C overnight. Excess VLP solution was removed, and the wells were blocked with 2.5% (wt/vol) skim milk in PBS supplemented with 0.05% (vol/vol) Tween 20 (PBST) for 2 h at room temperature with gentle shaking. Wells were washed with PBST and treated with 100 μl of the appropriate concentration of NEW for 30 s, after which 100 μl of D/E neutralization broth (10%, vol/vol) was added to terminate disinfection. After washing two more times with D/E broth, 100 μl biotinylated H type 2 HBGA (10 μg/ml; catalog number 01-019; GlycoTech, Gaithersburg, MD) diluted in 0.25% skim milk-PBST was added to each well and allowed to incubate for 1 h with gentle shaking. Wells were washed three times with PBST and subsequently incubated for 15 min with 100 μl of 1:5,000 diluted streptavidin-horseradish peroxidase conjugate (Invitrogen, Carlsbad, CA). Development was achieved using 100 μl/well TMB substrate solution (KPL, Gaithersburg, MD). Absorbance was read at 450 nm using an Infinite 200 Pro plate reader (Tecan, Morrisville, NC). Negative-control wells contained PBS only (no VLPs), and positive controls consisted of VLPs not receiving NEW treatment. The absorbance values of negative-control wells were subtracted from those of the positive-control and sample wells to normalize treatment absorbance. Binding reduction was calculated based on the percentage of normalized treated sample absorbance to that of the positive controls.

Data analysis. All quantitative experiments were performed in triplicate. Statistical comparisons between treatments were made with Tukey's honest significant difference (HSD) test using JMP

version Pro 12 (SAS Institute Inc., Cary, NC). A *P* value of less than 0.05 was considered statistically significant.

ACKNOWLEDGMENTS

We sincerely thank Tom Johnson from Johnson Risk Services for providing the water electrolyzing device and for insightful technical advice.

This work was supported by the Agriculture and Food Research Initiative Competitive grant no. 2011-68003-30395 from the U.S. Department of Agriculture, National Institute of Food and Agriculture through the NoroCORE project.

REFERENCES

1. Ahmed SM, Hall AJ, Robinson AE, Verhoef L, Premkumar P, Parashar UD, Koopmans M, Lopman BA. 2014. Global prevalence of norovirus in cases of gastroenteritis: a systematic review and meta-analysis. Lancet Infect Dis 14:725–730. https://doi.org/10.1016/S1473-3099(14)70767-4.
2. Scallan E, Hoekstra RM, Angulo FJ, Tauxe RV, Widdowson MA, Roy SL, Jones JL, Griffin PM. 2011. Foodborne illness acquired in the United States–major pathogens. Emerg Infect Dis 17:7–15. https://doi.org/10.3201/eid1701.P11101.
3. Lopman B, Gastanaduy P, Park GW, Hall AJ, Parashar UD, Vinje J. 2012. Environmental transmission of norovirus gastroenteritis. Curr Opin Virol 2:96–102. https://doi.org/10.1016/j.coviro.2011.11.005.
4. Otter JA, Yezli S, Salkeld JA, French GL. 2013. Evidence that contaminated surfaces contribute to the transmission of hospital pathogens and an overview of strategies to address contaminated surfaces in hospital settings. Am J Infect Control 41:S6–S11. https://doi.org/10.1016/j.ajic.2012.12.004.
5. Teunis PF, Sukhrie FH, Vennema H, Bogerman J, Beersma MF, Koopmans MP. 2015. Shedding of norovirus in symptomatic and asymptomatic infections. Epidemiol Infect 143:1710–1717. https://doi.org/10.1017/S095026881400274X.
6. Tung G, Macinga D, Arbogast J, Jaykus LA. 2013. Efficacy of commonly used disinfectants for inactivation of human noroviruses and their surrogates. J Food Prot 76:1210–1217. https://doi.org/10.4315/0362-028X.JFP-12-532.
7. Kotwal G, Cannon JL. 2014. Environmental persistence and transfer of enteric viruses. Curr Opin Virol 4:37–43. https://doi.org/10.1016/j.coviro.2013.12.003.
8. Cromeans T, Park GW, Costantini V, Lee D, Wang Q, Farkas T, Lee A, Vinje J. 2014. Comprehensive comparison of cultivable norovirus surrogates in response to different inactivation and disinfection treatments. Appl Environ Microbiol 80:5743–5751. https://doi.org/10.1128/AEM.01532-14.
9. United States Centers for Disease Control Prevention. 2011. Updated norovirus outbreak management and disease prevention guidelines. MMWR Recomm Rep 60:1–18.
10. Rutala WA, Weber DJ, Healthcare Infection Control Practices Advisory Committee. 2008. Guideline for disinfection and sterilization in healthcare facilities, 2008. United States Centers for Disease Control Prevention, Atlanta, GA.
11. Girard M, Ngazoa S, Mattison K, Jean J. 2010. Attachment of noroviruses to stainless steel and their inactivation, using household disinfectants. J Food Prot 73:400–404. https://doi.org/10.4315/0362-028X-73.2.400.
12. Pangloli P, Hung YC, Beuchat LR, King CH, Zhao ZH. 2009. Reduction of *Escherichia coli* O157:H7 on produce by use of electrolyzed water under simulated food service operation conditions. J Food Prot 72:1854–1861. https://doi.org/10.4315/0362-028X-72.9.1854.
13. Wang X, Dev S, Demirci A, Graves R, Puri V. 2012. Electrolyzed oxidizing water for cleaning-in-place of milking systems on dairy farms. Paper # NABEC/CSBE 12-063. Canadian Society for Bioengineering, Winnipeg, Manitoba, Canada. http://www.csbe-scgab.ca/docs/meetings/2012/CSBE12063.pdf.
14. Rahman SME, Khan I, Oh DH. 2016. Electrolyzed water as a novel sanitizer in the food industry: current trends and future perspectives. Compr Rev Food Sci Food Saf 15:471–490. https://doi.org/10.1111/1541-4337.12200.
15. Park GW, Boston DM, Kase JA, Sampson MN, Sobsey MD. 2007. Evaluation of liquid- and fog-based application of Sterilox hypochlorous acid solution for surface inactivation of human norovirus. Appl Environ Microbiol 73:4463–4468. https://doi.org/10.1128/AEM.02839-06.
16. Fang J, Cannon JL, Hung YC. 2016. The efficacy of EO waters on inactivating norovirus and hepatitis A virus in the presence of organic matter. Food Control 61:13–19. https://doi.org/10.1016/j.foodcont.2015.09.011.
17. American Society for Testing and Materials. 2011. Standard test method to assess the activity of microbicides against viruses in suspension. E1052-11. American Society for Testing and Materials International, West Conshohocken, PA.
18. American Society for Testing and Materials. 2011. Standard test method to assess virucidal activity of chemicals intended for disinfection of inanimate, nonporous environmental surfaces. E1053-11. American Society for Testing and Materials International, West Conshohocken, PA.
19. Knight A, Li D, Uyttendaele M, Jaykus LA. 2013. A critical review of methods for detecting human noroviruses and predicting their infectivity. Crit Rev Microbiol 39:295–309. https://doi.org/10.3109/1040841X.2012.709820.
20. Sattar S, Springthorpe S, Adegbunrin O, Zafer A, Busa M. 2003. A disk-based quantitative carrier test method to assess the virucidal activity of chemical germicides. J Virol Methods 112:3–12. https://doi.org/10.1016/S0166-0934(03)00192-7.
21. Mattle MJ, Crouzy B, Brennecke M, Wigginton KR, Perona P, Kohn T. 2011. Impact of virus aggregation on inactivation by peracetic acid and implications for other disinfectants. Environ Sci Technol 45:7710–7717. https://doi.org/10.1021/es201633s.
22. Sobsey MD. 1989. Inactivation of health-related microorganisms in water by disinfection processes. Water Sci Technol 21:179–195.
23. Samandoulgou I, Fliss I, Jean J. 2015. Zeta potential and aggregation of virus-like particle of human norovirus and feline calicivirus under different physicochemical conditions. Food Environ Virol 7:249–260. https://doi.org/10.1007/s12560-015-9198-0.
24. Randtke SJ. 2010. Chemistry of aqueous chlorine, p 91–103. *In* Black and Veatch Corporation (ed), White's handbook of chlorination and alternative disinfectants, 5th ed. John Wiley & Sons Inc., Hoboken, NJ.
25. Nowak P, Topping JR, Bellamy K, Fotheringham V, Gray JJ, Golding JP, Wiseman G, Knight AI. 2011. Virolysis of feline calicivirus and human GII.4 norovirus following chlorine exposure under standardized light soil disinfection conditions. J Food Prot 74:2113–2118. https://doi.org/10.4315/0362-028X.JFP-11-087.
26. Nuanualsuwan S, Cliver DO. 2002. Pretreatment to avoid positive RT-PCR results with inactivated viruses. J Virol Methods 104:217–225. https://doi.org/10.1016/S0166-0934(02)00089-7.
27. Page MA, Shisler JL, Mariñas BJ. 2010. Mechanistic aspects of adenovirus serotype 2 inactivation with free chlorine. Appl Environ Microbiol 76:2946–2954. https://doi.org/10.1128/AEM.02267-09.
28. Sano D, Pinto RM, Omura T, Bosch A. 2010. Detection of oxidative damages on viral capsid protein for evaluating structural integrity and infectivity of human norovirus. Environ Sci Technol 44:808–812. https://doi.org/10.1021/es9018964.
29. Farkas T, Sestak K, Wei C, Jiang X. 2008. Characterization of a rhesus monkey calicivirus representing a new genus of *Caliciviridae*. J Virol 82:5408–5416. https://doi.org/10.1128/JVI.00070-08.
30. Zhang D, Huang P, Zou L, Lowary TL, Tan M, Jiang X. 2015. Tulane virus recognizes the A type 3 and B histo-blood group antigens. J Virol 89:1419–1427. https://doi.org/10.1128/JVI.02595-14.
31. Park GW, Sobsey MD. 2011. Simultaneous comparison of murine norovirus, feline calicivirus, coliphage MS2, and GII.4 norovirus to evaluate the efficacy of sodium hypochlorite against human norovirus on a fecally soiled stainless steel surface. Foodborne Pathog Dis 8:1005–1010. https://doi.org/10.1089/fpd.2010.0782.

32. Jiang X, Wang M, Graham DY, Estes MK. 1992. Expression, self-assembly, and antigenicity of the Norwalk virus capsid protein. J Virol 66:6527–6532.
33. Jothikumar N, Lowther JA, Henshilwood K, Lees DN, Hill VR, Vinjé J. 2005. Rapid and sensitive detection of noroviruses by using TaqMan-based one-step reverse transcription-PCR assays and application to naturally contaminated shellfish samples. Appl Environ Microbiol 71:1870–1875. https://doi.org/10.1128/AEM.71.4.1870-1875.2005.
34. Escudero BI, Rawsthorne H, Gensel C, Jaykus LA. 2012. Persistence and transferability of noroviruses on and between common surfaces and foods. J Food Prot 75:927–935. https://doi.org/10.4315/0362-028X.JFP-11-460.
35. Manuel CS, Moore MD, Jaykus LA. 2015. Destruction of the capsid and genome of GII.4 human norovirus occurs during exposure to metal alloys containing copper. Appl Environ Microbiol 81:4940–4946. https://doi.org/10.1128/AEM.00388-15.