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Sanitizer Efficacy against Murine Norovirus, a Surrogate for Human Norovirus, on Stainless Steel Surfaces when Using Three Application Methods

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Human noroviruses are major etiologic agents of epidemic gastroenteritis. Outbreaks are often accompanied by contamination of environmental surfaces, but since these viruses cannot be routinely propagated in laboratory cultures, their response to surface disinfectants is predicted by using surrogates, such as murine norovirus 1 (MNV-1). This study compared the virucidal efficacies of various liquid treatments (three sanitizer liquids, 5% levulinic acid plus 2% SDS [LEV/SDS], 200 ppm chlorine, and an isopropanol-based quaternary ammonium compound [Alpet D2], and two control liquids, sterile tap water and sterile tap water plus 2% SDS) when delivered to MNV-1-inoculated stainless steel surfaces by conventional hydraulic or air-assisted, induction-charged (AAIC) electrostatic spraying or by wiping with impregnated towelettes. For the spray treatments, LEV/SDS proved effective when applied with hydraulic and AAIC electrostatic spraying, providing virus reductions of 2.71 and 1.66 log PFU/ml, respectively. Alpet D2 provided a 2.23-log PFU/ml reduction with hydraulic spraying, outperforming chlorine (1.16-log PFU/ml reduction). Chlorine and LEV/SDS were equally effective as wipes, reducing the viral load by 7.05 log PFU/ml. Controls reduced the viral load by <1 log with spraying applications and by >3 log PFU/ml with wiping. Results indicated that both sanitizer type and application methods should be carefully considered when choosing a surface disinfectant to best prevent and control environmental contamination by noroviruses.

Human noroviruses (HuNoV) are a major public health concern, now recognized as the most common cause of epidemic gastroenteritis globally (1). In the United States, 21 million illnesses due to HuNoV occur each year, with an estimated 5.5 million cases of food-borne illness (2). Norovirus gastroenteritis, marked by vomiting and/or nonbloody diarrhea, usually occurs approximately 24 to 48 h after infection and subsides within 1 to 5 days, although asymptomatic infections and prolonged shedding of virus in feces (up to 8 weeks postinfection) can occur (3). HuNoV has a low infectious dose (less than 100 viral particles) (1, 4) and is transmitted through the fecal-oral route. Environmental contamination has been implicated in outbreaks (5, 6), and viral RNA is often detected on environmental surfaces associated with outbreaks (7). One gram of feces can contain up to 10^{12} viral particles, and 30 ml of projectile vomit can potentially distribute up to 3×10^7 viral particles into the surrounding environment (3, 8). Following contamination, noroviruses can survive and remain infectious on fomite surfaces for 2 weeks or more (5, 9, 10). Improper surface disinfection and hand hygiene can contribute to the spread of virus to secondary surfaces via cleaning cloths and the hands of the person cleaning or others that come into contact with the contaminated surface (11, 12).

Routine laboratory culture of HuNoV is not yet possible. Feline calicivirus (FCV) and murine norovirus 1 (MNV-1) are commonly used as surrogates when testing disinfectants for efficacy against HuNoV (9, 13–16), but MNV-1is preferred when testing disinfectants with a low pH, due to the instability of FCV, particularly in solutions with a pH below 4.0 (16–18). Demonstration of the effectiveness of surface disinfectants against FCV is required by the U.S. Environmental Protection Agency (EPA) in order to register a surface disinfectant and for the manufacturer(s) to make claims that it is virucidal against noroviruses (13, 15).

Sanitizers with different modes of virucidal activity have been shown effective against FCV, including 200 ppm chlorine (19), Alpet D2 (Best Sanitizers, Inc., Penn Valley, CA) (20), and a levulinic acid plus sodium dodecyl sulfate (LEV/SDS) sanitizer (21). Chlorine-based sanitizers, with their broad-spectrum oxidative powers, are in widespread use as no-rinse sanitizers for food contact surfaces (22) and as such may contain up to 200 ppm chlorine, as approved by the U.S. Food and Drug Administration (FDA) (47). Alpet D2, a tincture of a quaternary ammonia-based disinfectant, includes isopropyl alcohol (58.6%) and quaternary ammonium compounds (octyl decyl dimethyl ammonium chloride, dodecyl dimethyl ammonium chloride, and dioctyl dimethyl ammonium chloride). It is registered with the EPA for use against noroviruses (15, 20) and FDA approved for use on food contact surfaces. LEV/SDS is an acid anionic sanitizer with demonstrated antimicrobial activity, even in the presence of organic matter (23–25). Levulinic acid and SDS are FDA-approved food additives (48, 49). While individually ineffective, the efficacies of combinations of LEV/SDS have been demonstrated against the norovirus surrogates MNV-1 and FCV (21).

There is a wide range of available spray disinfectants/sanitizers marketed for use in homes, medical institutions, and in food service/food-processing operations. Spray bottles or other hydraulic

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atomizing apparatuses (i.e., liquid forced by hydraulic pressure through a confined space or nozzle orifice to form spray droplets) are commonly used during disinfectant/sanitizer applications. However, droplet size and projection force vary between spraying systems. The amount of disinfectant/sanitizer delivered and coverage on the targeted surfaces depends upon droplet dimensions, and the kinetic energy (a function of the amount of pressure applied) of spray droplets affects the ability of the liquid to dislodge microorganisms from surfaces (26). Air-assisted, induction-charged (AAIC) electrostatic spray technology makes use of a pneumatic atomizing, electrostatic induction-charging nozzle to produce highly charged droplets of spray liquid with air-assisted delivery to target surfaces. While standard hydraulic sprays typically have a broad spectrum of droplet sizes with a median diameter of approximately 300 μm on a mass or a volume basis (27, 28), AAIC electrostatic spray devices deliver much smaller droplets (approximately 30 to 40 μm in diameter) (29). AAIC electrostatic spraying is commonly used by the sunless tanning industry (facilitating better, more even coverage of tanning solutions) (30), for pesticide spray applications (31), and for infectious disease outbreak prevention (32, 45). At the instant of their formation, AAIC droplets are adequately charged (typical charge-to-mass ratio of −6 to −12 mC/kg) to overcome gravitational forces during delivery (29). A lower volume of spray liquid is thus needed to achieve the desired surface coating, and a wraparound effect of charged liquid particles allows coverage of hidden areas, such as under or on backsides of target surfaces (33, 34). Lyons et al. found that AAIC electrostatic spraying increased the deposition of active sanitizing ingredient on the backside of a target surface 29-fold above that with a conventional, hydraulic spray application method (27, 28).

Wet wipes, or towelettes, presaturated with sanitizer are advantageous because they are ready and easy to use. They are often used for sanitizing on-the-go and where access to potable water is limited. Also, because the wipes are disposable, the potentials for pathogen spread and cross-contamination decline if they are used on one surface at a time. Furthermore, frictional force is generated during application, which can facilitate pathogen removal from surfaces. However, care must be taken when selecting this method for surface disinfection, because the contact time required for disinfection is often longer than is practical. For example, Clorox makes a disinfecting wipe that claims to kill 99.9% of rhinoviruses and influenza virus A2, but it requires the presaturated wipes to be applied to a surface to create a 4-min visibly wet contact time, and a rinse is required if the surface is a food contact surface (35). Also, because frictional force is user generated (and dependent on the pressure applied by hand), significant variability in a wipe's ability to remove viral load by mechanical action is likely.

The objective of this study was to compare the antinoroviral efficacies of various liquid treatments (three sanitizer liquids, LEV/SDS, 200 ppm chlorine, and Alpet D2, and two control liquids, sterile tap water and sterile tap water plus 2% SDS) when delivered to MNV-1-inoculated stainless steel surfaces by conventional hydraulic spraying, AAIC electrostatic spraying, or wiping with impregnated towelettes. It was hypothesized that the three different sanitizer liquids, having different active ingredients, would perform differently, depending on the method of application. In order to evaluate differences in the effectiveness of the sanitizers, any variability due to sanitizer delivery had to be carefully controlled. Therefore, engineering expertise was sought for the design and fabrication of the delivery systems used for the spray and wipe application methods so that variability between replicates could be minimized. The study results highlight the importance of selecting an appropriate combination of application method and surface sanitizer for maximal disinfection of surfaces contaminated with noroviruses.

MATERIALS AND METHODS

Virus cultivation and plaque assay. RAW 264.7 macrophage cells (ATCC TIB-71) were maintained in complete DMEM (Dulbecco's modified Eagle's medium; HyClone, Thermo Fisher Scientific, Inc., Logan, UT) containing 10% low-endotoxin fetal bovine serum (FBS; SH3007003; HyClone), 1% penicillin (100 U/ml)–streptomycin (100 U/ml) (HyClone), 1% 1 M HEPES buffer (Lonza, Biowhittaker, Alpharetta, GA), and 1% 100 mM sodium pyruvate (Cellgro, Mediatech, Inc., Manassas, VA) and passaged every 2 to 3 days. Confluent (80 to 90%) monolayers of cells were infected with MNV-1 (obtained from H. Virgin, Washington School of Medicine) for approximately 48 h at 37°C in a 5% CO_2 environment. For stock preparation, virus was harvested after complete cytopathic effect was apparent via three cycles of freeze-thawing. MNV-1 was centrifuged at 2,000 × *g* for 15 min at 20°C and filtered using a 0.2-μm membrane filter (Nalgene, Rochester, NY). To concentrate the virus stock, this partially purified cell culture lysate was ultracentrifuged at 100,000 × *g* for 1 h at 4°C, and the pellet was resuspended overnight in 1/10 the original volume in phosphate-buffered saline (PBS; 8 g/liter NaCl, 0.2 g/liter KCl, 0.12 g/liter KH_2PO_4, 0.91 g/liter Na_2HPO_4, with pH adjusted to 7.4) containing 5% (vol/vol) FBS. One-milliliter portions of MNV-1 were stored at −70 ± 2°C (Innova U535 ultra-low temperature freezer; New Brunswick Scientific, Edison, NJ) until used.

A standard plaque assay was performed to quantify viral infectivity, as previously described (21, 36). Briefly, cells were grown to 80 to 90% confluence on 60-mm by 15-mm tissue culture plates (CellStar; Greiner Bio-One, Monroe, NC) containing 5 ml complete DMEM. Medium was replaced with 400 μl of infection medium (1× modified Eagle's medium [MEM; Cellgro] containing 1% 1 M HEPES buffer, 1% penicillin and streptomycin, 1% 100 mM sodium pyruvate, 5% HyClone FBS) and 100 μl of either undiluted or serially diluted sample (in 1× MEM), in duplicate. Plates were incubated for 1 h at 37°C with 5% CO_2, and trays of cells were rocked every 15 min to allow virus adsorption to cells. At the end of the hour, the liquid portion was aspirated, and cells were overlaid with 3 ml of 1× MEM containing 0.5% agarose (SeaKem LE agarose; Lonza, Rockland, ME) and incubated for 48 ± 5 h at 37°C with 5% CO_2 to allow for virus infection. A second agarose overlay consisting of 3 ml of 0.75% agarose with 1.1% neutral red solution (from a 3.3-g/liter stock solution; Sigma-Aldrich, St. Louis, MO) was administered to each plate after 48 h, and plaques were counted 4 to 8 h later to determine infectious PFU. Duplicate negative controls with and without agarose, and duplicate positive controls, with and without agarose, were included to ensure plaque assay function.

Virus inoculation onto stainless steel surfaces. Sterile stainless steel coupons (5 cm by 2 cm by 1 mm; type 304, Finish 4) were washed and scrubbed with mild detergent and tap water, dried with paper towels, soaked in 70% ethanol for 1 h with occasional vigorous shaking, rinsed three times with sterile Milli-Q water, dried with wipes (Kimwipes; Kimberly-Clark, Neenah, WI), autoclaved at 121°C for 30 min, and stored in a sterile container until use. Coupons were then spot inoculated with five 15-μl portions (75 μl, total volume) of MNV-1 virus stock or PBS containing 5% (vol/vol) FBS, which servesd as a negative control. High-titer MNV-1 virus stock ranged from approximately 6 to 8 log PFU MNV-1/ml. The inoculum was then evenly spread over each coupon by using a pipette tip to create a thin liquid surface layer. Coupons were allowed to dry on the benchtop of a biosafety level 2 laboratory room for at least 50 min or until visibly dry. Timing of the coupon inoculation was carefully orchestrated to ensure that once visibly dry, coupons were treated within 5 min. Five inoculated coupons per experimental replicate were included

TABLE 1 Properties of sanitizers used on stainless steel surfaces for disinfection of norovirus

Sanitizer	Active ingredient(s)	pH	Electric conductivity (μS/cm)	Inactivation of FCV[a] (reference)
Levulinic acid + SDS	Low pH (below 4.0) and SDS	2.58–2.79	3,650–3,710	21
200 ppm chlorine	200 ppm free available chlorine	8.80–9.16	981–1,420	19
Alpet D2	Isopropyl alcohol (58.6%) containing quaternary ammonium compounds	6.93	10.0–10.3	20

[a] Listed is the study that demonstrated the corresponding sanitizer's effectiveness against FCV.

as no-treatment, recovery controls. Relative humidity and ambient temperature during drying ranged from 26 to 63% and 20.5 ± 1.3°C, respectively.

Preparation of sanitizers and controls. SDS (Sigma-Aldrich Co., St. Louis, MO), levulinic acid (98% solution; Sigma), and household bleach (6.25% sodium hypochlorite; Inter-American Products, Inc., Cincinnati, OH) were mixed with sterile tap water from one single laboratory source to formulate test liquids in the study. On each day of use, solutions of 2% SDS, 5% LEV plus 2% SDS (LEV/SDS), and 200 ppm (free) chlorine were prepared. Free, available chlorine was measured using a chlorine titration kit (iodometric, starch-iodide method; chlorine test kit 101; Ecolab Center, St. Paul, MN) to ensure 200 ppm free chlorine accuracy in 10 ppm increments. Alpet D2 was purchased from Best Sanitizers, Inc. (Penn Valley, CA) and used without dilution per the manufacturer's instructions. Measurements of pH, water hardness (test kit 402; Ecolab Center, St. Paul, MN), and electric conductivity (meter HI 8733; Hanna Instruments, Ann Arbor, MI) were taken and recorded as appropriate. Water hardness values ranged from 34 to 68 ppm throughout all experimental replicates, indicating that the water used was not hard water. Table 1 lists the active ingredients, pH range, and electric conductivity of the sanitizers used in this study. These sanitizers were chosen because they had been previously shown to be effective against the FCV surrogate for HuNoV, as referenced in Table 1.

Neutralization of sanitizer liquids and elution of virus from stainless steel coupons. A novel "neut/elute combo" comprised of letheen broth with 5 g/liter Tween 80 (Neogen Corporation, Lansing, MI) plus 1 M NaCl, 0.02 g sodium thiosulfate/liter, and 4.0 g sodium bicarbonate/liter was used to neutralize all three sanitizer liquids while simultaneously eluting virus from the stainless steel coupon surfaces. Each experimental replicate included five negative-control stainless steel coupons, which were mock inoculated with 75 μl of PBS containing 5% FBS instead of virus. After each of the 5 liquid treatments (sanitizers and control solutions), the coupons were each placed into a 50-ml tube containing 15 ml of neut/elute combo for sanitizer neutralization. A portion of each was then used as a cytotoxicity control, and 11.94 ml of each was used for a neutralization control. For neutralization controls, 60 μl of virus stock was added to each tube and incubated for 5 min before portioning into 0.5-ml aliquots and storage at −80°C. Cytotoxicity controls ensured that the neutralization solution alone, or with the sanitizers, did not cause any cytotoxic effects on the cell cultures. Posttreatment, each stainless steel target coupon immediately was placed into a sterile 50-ml centrifuge tube (Corning, Inc., Corning, NY) containing 15 ml of neut/elute combo at room temperature. Each tube was inverted gently three times to allow the liquid to contact the surface of the coupon completely before vortexing at full speed for 30 s (Vortex-Genie 2; VWR, Radnor, PA). Samples were aliquoted into two 500-μl portions for storage at −80 ± 2°C for at least 4 h before the plaque assay. The remaining (~13 ml) liquid from each sample also was stored at −80 ± 2°C until future use if necessary. Freezing the samples one time only was a way to reduce variability that may have been caused by immediately conducting some experiments and having to freeze-thaw other samples before plating, if the cell cultures looked unhealthy or not ready on the day of the experiment.

MNV-1 concentrated by PEG precipitation. For 19 samples (of the wipe application experiments) for which no infectious virus could be detected on the zero dilution plates, a polyethylene glycol (PEG) precipitation method was used to lower the assay limit of detection. In this procedure, 8% (wt/wt) PEG 8000 (Sigma-Aldrich, Co., St. Louis, MO) was added to each sample in 50-ml centrifuge tubes, which were vortexed immediately for approximately 5 s. Tubes were placed in a shaking rack (200 rpm), and PEG was dissolved overnight at 4°C. The following day, samples were centrifuged at 9,000 × *g* for 30 min at 4°C. Subsequently, the supernatant was poured off, and the resulting pellet was suspended in 1 ml of 0.1 M NaCl–PBS (pH 7.4) with vortexing. Samples were divided into two 500-μl portions and frozen to −80 ± 2°C for at least 4 h before assaying.

Carrier method for treating stainless steel coupons with surface disinfectants. A "carrier method," similar to the U.S. EPA Confirmatory Virucidal Effectiveness Test (13), was employed to test the efficacy of each sanitizer solution against MNV-1 on stainless steel. Briefly, stainless steel coupons inoculated with MNV-1 were placed in the center of the bottom portion of a sterile glass petri dish (100 by 15 mm). Test liquids (sanitizers and controls) were gently pipetted into the petri dishes in 15-ml portions so that the stainless steel coupons were completely submerged in the liquid solution for 5 min at room temperature. Coupons were then removed from the liquid by carefully gripping the noninoculated portions of the stainless steel coupons at the edges with clean gloved finger tips, changing gloves between each sample. They were then held at room temperature for 1 min before neutralization and elution using the neut/elute combo.

Conventional hydraulic spray application. All spray applications were implemented in a University of Georgia laboratory facility developed and briefly described by Lyons et al. (27, 28). A more detailed description is reported here. Inside a biosafety level 2 laboratory, a stainless steel smokehouse oven (model 450; Alkar-RapidPak, Inc., Lodi, WI) was modified into a robotically controlled spray chamber (Fig. 1). The smokehouse door was replaced at the bottom with a plastic dam 2.54 cm in height to

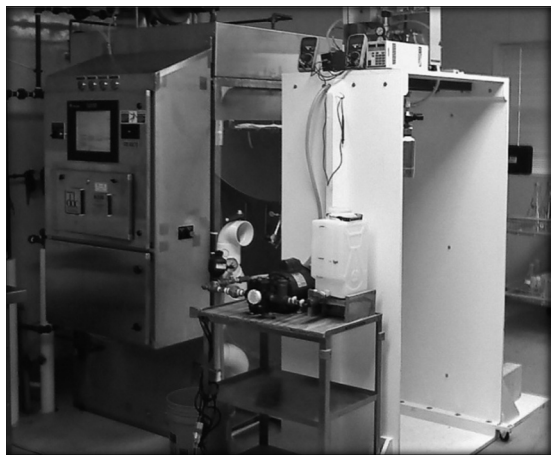

FIG 1 Spray chamber (side view) converted for hydraulic spray and AAIC electrostatic spray application methods fabricated from a stainless steel smokehouse oven.

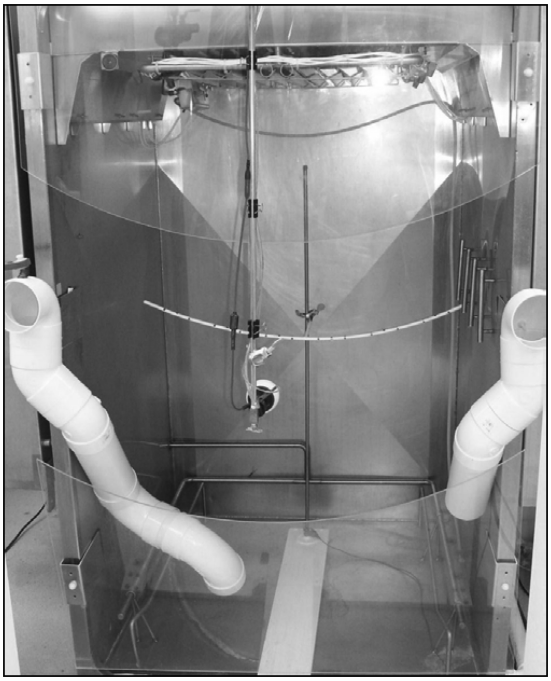

FIG 2 Front view of spray chamber used for hydraulic and AAIC electrostatic spray application methods, showing the spray nozzle and arched attachment point for target coupons.

FIG 3 Arched attachment point for virus-inoculated stainless steel target coupons, secured in place by alligator clips inside the spray chamber.

contain liquid runoff and with two large acrylic facings framing an opening for a spray cloud to enter in a sweeping-arc motion, thereby maximizing the exhaust function of the smokehouse and minimizing environmental exposure to spray applications. The exhaust fan provided approximately 0.28 m^3/min of constant, upward airflow and remained in the on position during all replicates. The electrically grounded spray chamber was 1.5 m high by 1 m wide by 1 m deep and was fitted with a tall ring stand holding a metal arch at a height of 83 cm, which allowed target coupons to be attached radially with sterilized alligator clips (Ideal Industries, Inc., Sycamore, IL). The target-holding metal arch had four equidistant holes spaced 15.24 cm apart with thumb screws to affix the alligator clips (Fig. 2). This setup allowed four coupons (3 MNV-1-inoculated coupons plus 1 PBS/FBS-inoculated coupon, serving as a negative control) to be sprayed at one time with each test liquid for each replicate (Fig. 3). The tall ring stand was also attached to a horizontal sliding mechanism so that the spraying nozzle-to-target coupon distance could be adjusted to provide the same spray swath width (30.5 cm) at the coupons for all nozzle types. For this hydraulic application, the distance between the spraying nozzle tip and the target coupon was 42 cm, to provide the appropriate swatch width at the target coupons, as previously defined (27).

The hydraulic spraying apparatus consisted of a liquid-holding reservoir, a pump and motor, a bypass type pressure regulator with gauge, and tubing leading to a robotic arm with the hydraulic nozzle attached to the end. A plywood rack held the apparatus. The test liquid was contained in an 8-liter plastic reservoir (Nalgene, Rochester, NY) with an on/off valve, which was connected to a twin piston pump (Dayton model 6AWC3) to deliver a nozzle liquid pressure of 295 kPa as measured with a gauge (Weksler, Deer Park, NY) with excess by-passed liquid returned to the reservoir.

Test liquid flowed from the pump through plastic tubing into a 0.635-cm-diameter stainless steel, tubular robotic arm that was 0.99 m in length. Attached perpendicular to the distal end of the rotating arm steel tube was the conventional hydraulic atomizing spray nozzle (Teejet Even flat spray tip TP40015E with strainer 4514-NY-20; Spraying Systems Co., Springfield, IL). The volume median diameter of the spray droplets created was approximately 300 μm, and the flow rate was 600 ml/min. The slotted orifice tip of the spray nozzle was positioned parallel to the robotic arm, creating a 30.5-cm-wide flat fan spray pattern that passed at 76 cm/s over the coupons from left to right and then from right to left in one dual-pass sweep with a 120° spray arc (spatially aligned in sync with the metal targets on the coupon-holding arch), with spraying lasting 6,000 ± 200 ms. An electronic controller and digital timer facilitated an accurate and repeatable traverse time of the robotic arm-mounted nozzle.

One liter of each test liquid was allowed to flush through the setup before being applied to the coupons, and it was continuously stirred during application by using a magnetic stir bar and a stir plate (VWR, Radnor, PA) underneath the reservoir. A sterile tap water rinse of at least 2 liters was utilized in between the LEV/SDS and 200 ppm chlorine and the 200 ppm chlorine and Alpet D2 treatments, but not between the sterile tap water, sterile tap water plus SDS, or LEV/SDS treatments, because the treatments were applied in the order stated. Coupons were sprayed and allowed a 5-min treatment contact time (starting with the first pass) plus a 1-min allowance in the chamber for air drying before being placed into the neut/elute combo solution.

AAIC electrostatic spray application. The same spray chamber and electronic-controlled robotic arm as used for the hydraulic spray application was used for the AAIC electrostatic spray application, but with a different liquid reservoir/tubing system and nozzle. A commercial pneumatic atomizing, electrostatic spray nozzle (MaxCharge; Electrostatic Spraying Systems, Inc., Watkinsville, GA) was used, as developed by Law (29) and patent licensed by The University of Georgia Research Foundation to ESS, Inc., for technology transfer. Both induced charge and pneumatic energy act in concert to create finely atomized, highly charged spray droplets with a volume median diameter of approximately 30 to 40 μm. Compressed air, controlled at 207 kPa by a pressure regulator and measured with a pressure gauge (Weksler, Deer Park, NY), flowed through the steel robotic arm to pneumatically atomize the spray liquid. The test liquid was drawn by venture suction through plastic tubing into the nozzle. Energy for droplet charging came from a low-voltage power supply (model LLS 6018; TDK-Lambda Americas, Inc., San Diego, CA) was set between 6.65 and 11.00 Vdc, then stepped up to between 1,000 and 1,400 Vdc with a Venus high-voltage power supply (0- to 12-Vdc input; ITech Instruments, Châteauneuf-Les-Martigues, France) to provide voltage to the embedded induction electrode inside the spray nozzle, which in turn imparted a charge flow of −7.2 mC/kg onto the conductive liquid of the water-based spray cloud (except for the low-conductivity, alcohol-based Alpet D2, which only reached a charge flow of approximately −3.75 mC/kg). A digital multimeter fitted with a 26-gauge needle ionization probe,

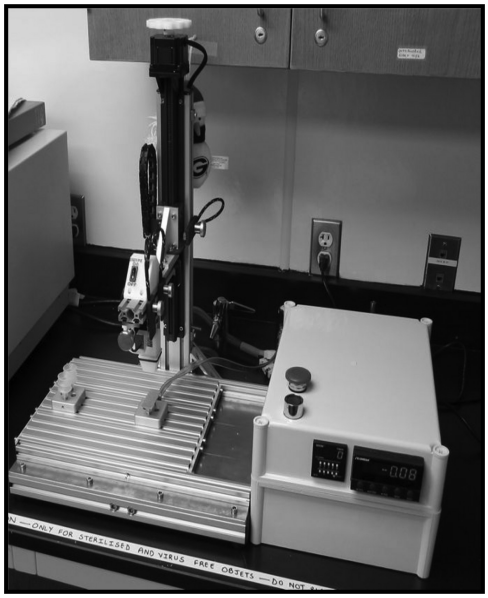

FIG 4 Robotic SAM fabricated for the wipe application method using premoistened towelettes.

held on the center line of the spray cloud at 2 to 3 cm from the nozzle face (model 410; Extech, Waltham, MA) measured and verified the charge convected on the spray before each test run. From the time rates of charge conveyed on the spray cloud and the liquid flow, which was measured and stabilized at 100 ml/min by using a flow rate meter (Key Instruments, Trevose, PA), the average charge-to-mass ratio for the AAIC electrostatic spray droplets was calculated to be −7.2 mC/kg (except for Alpet D2, which had a lower charge-to-mass ratio of only −3.75 mC/kg).

Instead of spraying the coupons at a nozzle-to-target coupon distance of 42 cm as with the hydraulic application, the distance for the AAIC electrostatic application was increased to 76 cm to provide an identical swath width (30.5 cm) at the target coupons. Also, since the treatment flow rate for the low-volume AAIC electrostatic application was only 100 ml/min while the hydraulic application was 600 ml/min, the coupons were sprayed with the dual pass six consecutive times in order to dispense toward each unit area of target coupons an equal mass of test liquid, ensuring unbiased comparisons independent of nozzle type.

The 5-min contact time began with the first pass, and coupons remained in the chamber for a 1-min air drying time allowance and then were immediately placed into the neut/elute combo solution.

Saturated wipe application. A robotic wipe machine, the Swiper Automated Machine (SAM; Engineerable LLC, Atlanta, GA), was constructed for this project (Fig. 4) (27). Briefly, custom wipe adapters (manufactured using an UP! 3D printer; Delta Micro Factory Corp., Beijing, China) were held in place by using neodymium magnets (Fig. 5) to a 2-kg single-point-load cell (Transducer Techniques, Inc., Temecula, CA) attached to a two-axis robotic arm. The robotic arm consisted of a horizontal and vertical axis driven by linear actuators (Haydon Kerk Motion Solutions, Waterbury, CT). Set points for the downward force delivered while wiping across the horizontal target coupon were digitally programmed and displayed using a weigh scale panel meter (Omega Engineering, Inc., Stamford, CT). The distances traveled by the wipe adaptors were manually set by end stop microswitches (Panasonic Electric Works, New Providence, NJ). A constant 9.8 N downward force (imposed by a 1,000 g ± 10 g mass load) at an overall horizontal speed of 0.56 cm/s over an area of 4 cm^2 (0.10 Pa) was sprayed back and forth over a single, horizontally positioned, stationary stainless steel test coupon. A T-slot aluminum extrusion panel (Misumi USA, Schaumburg, IL) provided an adjustable attachment point for the stainless steel target coupons, which

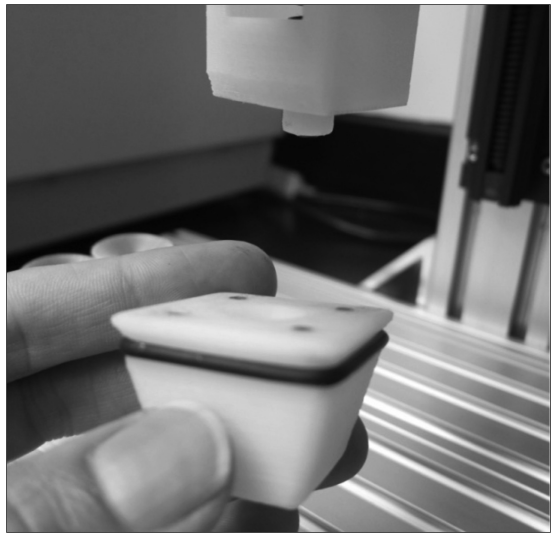

FIG 5 Close-up view of the removable adaptor for attaching wipes to the robotic arm of a SAM.

were held in place by two silicon suction cups (Anver Corp., Hudson, MA) and a custom vacuum manifold (Fig. 6).

Sterilized scissors were used to cut 24.1-cm by 18.3-cm dry towelettes (100% polyester; Best Sanitizers, Inc., Penn Valley, CA) into four equal rectangles, each 6.03 cm by 4.58 cm (the "wipes"). Treatment liquid solutions were prepared as mentioned previously in sterile 300-ml glass jars. The saturation volume for each wipe, 2.6 ml of sanitizer or control liquid, was placed into a sterile 15-ml centrifuge tube (VWR, West Chester, PA) for each sample plus one negative control per treatment per replicate. Wipes were folded in half four times, then fully submerged into the sanitizer or control liquid at the bottom of the tube 5 min prior to wiping using sterile forceps (Sigma-Aldrich, Co., St. Louis, MO), so as to saturate the entire wipe.

A wipe saturated with a treatment solution (or impregnated towelette) was held onto the plastic cube with an elastic band. The surface area of the wipe that came into contact with the coupon was 2 cm by 2 cm (4 cm^2). Contact time with the coupon was approximately 9 s per swipe, with a total of 18 s for the back and forth motion. A time allowance of 5 min plus 1 min of air drying was allotted, beginning with the first swipe. Subsequently, coupons were immediately placed into the neut/elute combo solution.

FIG 6 Close-up view of a stainless steel target coupon held in position by vacuum on the stationary platform of a vacuum on the stationary platform of a SAM.

TABLE 2 Recovery of infectious MNV-1 from inoculated stainless steel coupons treated with sanitizer liquids or control liquids[a]

Application method	Avg (SD) recovery (log PFU/ml) of MNV-1 from stainless steel coupons		
	Rep 1	Rep 2	Rep 3
Hydraulic spray	6.95 (0.19)	6.95 (0.04)	7.72 (0.06)
AAIC electrostatic spray	7.62 (0.13)	6.27 (0.06)	5.93 (0.06)
Robotic wiping	7.83 (0.12)	8.13 (0.09)	8.42 (0.04)

[a] Control liquids (recovery controls) were used for each experimental replicate (Rep) by the hydraulic spray, AAIC electrostatic spray, and robotic wiping application methods.

Calculation of log infectious virus reduction and statistical analysis. The lower limit of detection was 3.30 logs of virus, or 2,000 viral PFU/ml, for all replicates with the hydraulic, AAIC electrostatic, and wipe applications. As mentioned previously, this detection limit was lowered to 1.10 log PFU/ml of virus with the PEG precipitation procedure, which was performed for some treatments of the wipe experiment when no virus was detected on the zero dilution plate.

For each application method, sanitizer liquids and control liquids were prepared and applied on three different days to each of three test samples and negative controls. Average MNV-1 log reductions (in PFU/ml) due to each treatment were calculated using the average log MNV-1 (PFU/ml) of recovery controls as the baseline for each experimental replicate. The log PFU MNV-1/ml recovered posttreatment for each sample in each replicate was subtracted from the average log PFU MNV-1/ml recovered from recovery controls. Data were statistically analyzed by two-way analysis of variance (ANOVA; SAS 9.3; SAS Institute, Cary, NC), with treatment, day, and their interaction as factors, used to draw overall conclusions from the treatment-induced log reduction means for each application method and Tukey-Kramer adjustment for multiple comparisons. A generalized linear mixed model was assumed with a variance components covariance structure for random effects to account for dissimilar variances across the treatments. Differences between treatment log reduction means were considered significant when the *P* value of the difference was less than 0.05.

RESULTS

MNV-1 inactivation on stainless steel using the carrier method for sanitizer treatment. The three sanitizer liquids with different active ingredients that were chosen in this study were previously shown to be effective against the FCV surrogate for HuNoV, as referenced in Table 1. In this study, the effectiveness of each for inactivating MNV-1 on stainless steel was evaluated using the carrier method for assessing virucidal effectiveness. After neutralization and virus recovery using the neut/elute combo, no MNV-1 was recovered from coupons treated with LEV/SDS, 200 ppm chlorine, or Alpet D2 in preliminary tests of effectiveness for inactivation (data not shown). However, the average recoveries of MNV-1 from stainless steel coupons were 6.24 (±0.46) log PFU MNV-1/ml after treatment with sterile tap water and 5.97 (±1.01) after treatment with sterile tap water containing 2% SDS (means ± standard deviations). Neutralization controls conducted simultaneously indicated that all sanitizer liquids could be sufficiently neutralized using the neut/elute combo; however, after treatment with Alpet D2, a 1-min drying time (required for isopropanol evaporation) was needed after removing the coupons from the sanitizer for complete neutralization. Due to this, a 1-min incubation period where the coupons were placed on a clean paper towel (with the inoculated side facing up) after sanitizer treatment and before neutralization was incorporated after all test liquid treatments with the various application methods described herein.

TABLE 3 Average reductions of infectious MNV-1 for control (sterile tap water) and sanitizer liquids applied with a conventional hydraulic spray apparatus

Test solution	Avg (SD) log PFU MNV-1/ml reduction due to treatment in replicate expt:			
	1	2	3	Mean (of the 3 expts)[a]
Sterile tap water	0.50 (0.34)	1.10 (0.31)	1.01 (0.02)	0.87^{CD}
Sterile tap water plus 2% SDS	0.45 (0.09)	1.01 (0.34)	1.09 (0.14)	0.85^{D}
LEV/SDS	3.45 (0.35)	1.79 (0.22)	2.88 (0.23)	2.71^{A}
200 ppm chlorine	0.87 (0.21)	1.16 (0.24)	1.46 (0.09)	1.16^{C}
Alpet D2	2.29 (0.12)	2.94 (0.46)	1.46 (0.24)	2.23^{B}

[a] Different uppercase letters (A through D) indicate statistically significant differences in the means (of 3 replicates) for MNV-1 log reductions after liquid treatment.

Recovery of MNV-1 from untreated stainless steel coupons. To account for variation in the virus stock titers used on the day for each experimental replicate, recovery controls (stainless steel coupons inoculated with MNV-1 but not subjected to treatment) were conducted with each experimental replicate. The log PFU/ml recovery of MNV-1 from untreated stainless steel coupons was determined simultaneously with each replicate ($n = 5$) of the hydraulic spray, AAIC electrostatic spray, and robotic wipe application methods, as listed in Table 2. Recovery controls were considered when constructing statistical models for comparing average MNV-1 PFU/ml log reductions due to liquid treatment within each application method tested.

MNV-1 inactivation on stainless steel following sanitizer liquid application by hydraulic spray. Overall, the LEV/SDS sanitizer resulted in a significantly greater reduction in MNV-1 than all other sanitizers ($P < 0.02$) using hydraulic spray, as shown in Table 3. The average ($n = 9$) log PFU MNV-1/ml reduction due to LEV/SDS was 2.71. Alpet D2 sanitizer treatment resulted in a significantly greater reduction of MNV-1 than all other treatments except LEV/SDS ($P < 0.001$), with an average ($n = 9$) log PFU MNV-1/ml reduction due to Alpet D2 of 2.23. The average ($n = 8$) log PFU MNV-1/ml reduction for 200 ppm chlorine, which is in widespread use in the food industry as a spray sanitizer (22), was 1.16. Sterile tap water and sterile tap water plus 2% SDS did not significantly differ in average ($n = 9$) log PFU MNV-1/ml reductions, indicating that the addition of the SDS surfactant to sterile tap water did not increase its ability to remove MNV-1 from the surface under the conditions of this experiment.

MNV-1 inactivation on stainless steel following sanitizer liquid application by air-assisted, induction-charged electrostatic spray. An overall comparison between liquid treatments is shown in Table 4. The largest MNV-1 reductions were observed after treatment with LEV/SDS or 200 ppm chlorine, which were both significantly greater than all other treatments ($P < 0.005$). However, there was no significant difference in MNV-1 log reductions observed after treatment with LEV/SDS or 200 ppm chlorine ($P = 0.57$). Treatment with Alpet D2, sterile tap water plus 2% SDS, and sterile tap water were less effective. Alpet D2 had no greater ability to reduce infectious MNV-1 viral load under the conditions of this experiment than did sterile tap water; furthermore, it reduced the viral load to a significantly lesser extent than sterile tap water plus 2% SDS.

TABLE 4 Average reductions of infectious MNV-1 due to control and sanitizer liquid test solutions for treatments applied with AAIC electrostatic spray technology

Test solution	Avg (SD) log PFU MNV-1/ml reduction due to treatment in replicate expt:			
	1	2	3	Mean (of the 3 expts)[a]
Sterile tap water	−0.41 (0.03)	0.23 (0.34)	0.36 (0.31)	0.06[BC]
Sterile tap water plus 2% SDS	0.35 (0.22)	0.85 (0.03)	−0.27 (0.03)	0.31[B]
LEV/SDS	0.48 (0.21)	2.63 (0.70)	1.87 (1.08)	1.66[A]
200 ppm chlorine	−0.08 (0.10)	2.41 (0.96)	1.15 (0.58)	1.16[A]
Alpet D2	−0.15 (0.31)	−0.04 (0.04)	0.01 (0.13)	−0.06[C]

[a] Different uppercase letters (A to C) indicate statistically significant differences in the means (of 3 replicates) for MNV-1 log reductions after liquid treatment.

TABLE 5 Average reductions of infectious MNV-1 for control and sanitizer liquid test solutions applied by a robotic wiping device

Test solution	Avg (SD) log PFU MNV-1/ml reduction due to treatment in replicate expt:			
	Rep 1	Rep 2	Rep 3	Mean (of the 3 expts)[a]
Sterile tap water	4.01 (0.30)	3.32 (0.38)	3.51 (0.09)	3.61[B]
Sterile tap water plus 2% SDS	4.06 (0.28)	3.25 (0.39)	3.28 (0.30)	3.53[B]
LEV/SDS	6.75 (0.00)	7.05 (0.00)	7.34 (0.00)	7.05[A]
200 ppm chlorine	6.75 (0.00)	7.05 (0.00)	7.34 (0.00)	7.05[A]
Alpet D2	2.99 (0.47)	3.52 (1.32)	4.88 (1.50)	3.80[B]

[a] Different uppercase letters indicate statistically significant differences in the means (of 3 replicates) for MNV-1 log reductions after liquid treatment.

Day-to-day variation was apparent with AAIC electrostatic spray application (data not shown). For example, on day 3, 200 ppm chlorine was not significantly different from sterile tap water ($P = 0.31$). Also, on day 1, LEV/SDS and 200 ppm chlorine did not result in a significantly greater reduction than the other treatments ($P > 0.32$). Day 1 resulted in the most statistically significant differences between the treatments compared with the other days (on day 1, only the two pairs Alpet D2 versus sterile tap water plus 2% SDS [$P = 0.007$] and sterile tap water versus sterile tap water plus 2% SDS [$P = 0.001$] were significantly different from one another). After reviewing experimental conditions during the AAIC electrostatic replicates and due to the statistically significant differences in treatments overall, it is possible that the lower titer virus stock used for days 2 and 3 of the AAIC electrostatic replicates (see Table 2) increased the sanitizer treatment effectiveness for all treatments except sterile tap water plus 2% SDS.

MNV-1 inactivation on stainless steel following sanitizer liquid application by using saturated wipes. The LEV/SDS and 200 ppm chlorine treatments resulted in significantly greater MNV-1 reductions than the three other treatments for the robotic wipe application ($P < 0.0001$), as shown in Table 5. In fact, these two treatments both resulted in an average ($n = 9$) log PFU MNV-1/ml reduction of 7.05, which was the maximum reduction that could be determined given the assay limit of detection (including the PEG precipitation procedure). The other three treatments, sterile tap water, sterile tap water plus 2% SDS, and Alpet D2, resulted in overall average ($n = 9$) log PFU MNV-1/ml reductions of 3.61, 3.53, and 3.80, respectively, and were not statistically different from one another ($P > 0.97$). Viral reductions of approximately 3 log PFU of MNV-1/ml occurred with control treatments alone, indicating that the mechanical action of wet wiping is a potentially important tool for viral removal. The Alpet D2 sanitizer treatment did not prove to be more effective than sterile tap water if used as a wipe and, likewise, adding SDS surfactant to sterile tap water did not increase viral reduction for this application under the conditions of this study.

DISCUSSION

This study revealed variations in the virucidal efficacies of three sanitizers, LEV/SDS, 200 ppm chlorine, and Alpet D2, against a human norovirus surrogate, MNV-1, on a stainless steel surface when applied by three different application methods that included hydraulic spraying, air-assisted, induction-charged electrostatic spraying, and mechanical wiping with an impregnated towelette. All sanitizers tested have been shown to be effective against FCV in solution (19–21), but the MNV-1 surrogate was chosen for this study due to the acid sensitivity of FCV and acid resistance of MNV-1 (16), as well as the low pH of the LEV/SDS sanitizer. The application methods were designed and fabricated after consultation with engineer collaborators (E. Law, The University of Georgia, for the conventional hydraulic and AAIC electrostatic spray applications and D. Bauen, Engineerable LLC, for the SAM robotic wiping device). The devices were designed to consistently deliver the sanitizer liquids to the virus-inoculated stainless steel surfaces, thereby minimizing variability due to human and/or mechanical experimental error. By doing this, differences in mean log PFU/ml reductions of MNV-1 due to the sanitizer or control liquids could be more accurately determined. While we noted that there was significant day-to-day variation in the log PFU MNV-1/ml reductions observed within each application method group, this variation was likely due to differences in the initial titers of the virus stocks used each day, or differences in the ambient conditions under which the MNV-1 was dried onto the stainless steel coupons. Evidence that the mechanical devices contributed minimally to the experimental variability can be seen in Tables 3 to 5. The standard deviations within each replicate rarely exceeded 0.5. There were, however, a few exceptions, likely due to incompatibility between sanitizer liquid and method of delivery.

For example, minimal viral load reductions and high variability among replicates were observed following application of Alpet D2-saturated wipes. The evaporative nature of this quaternary ammonia-based tincture (containing 58.6% isopropanol) is consistent with these findings, making it less than ideal for use on sanitizing wipes for the removal of norovirus (Best Sanitizers, Inc., manufactures wipes impregnated with Alpet D2, but these carry no norovirucidal claims). AAIC electrostatic spray application of Alpet D2 was less effective than the hydraulic spray application, likely due to an experimental design flaw in selecting a non-aqueous-based sanitizer liquid for electrostatic spraying. Alpet D2 sanitizer dispensed from the AAIC electrostatic apparatus was produced from a spray liquid in the lower level of the desired electric conductivity range generally considered valid for the induction-charging process (27, 29, 37). The rate of charge conveyed on its 100-ml/min spray cloud reached a maximum of −5.2 μA, although the desired charge was −12 μA as routinely achieved with earlier-reported water-based sanitizer sprays (27). Addition of ions or lecithin to the Alpet D2 formulation may have increased

the charge on the spray cloud. However, the flammability of Alpet D2 makes it less than ideal for use in AAIC electrostatic spraying for safety reasons.

Overall, the LEV/SDS sanitizer performed either as well as or outperformed the other sanitizers tested using the three application methods in this study. MNV-1 resistance to independent treatment with LEV or SDS was previously demonstrated with short contact times (≤5 min) (21). The isoelectric points (pI) of human norovirus capsids are estimated to be between 5.5 and 6.0 for GI and GII norovirus (38), which is similar to the theoretical pI of the Norwalk virus major capsid protein (NCBI accession number M87661.2), estimated to be 5.64 using the ExPASy ProtParam tool (http://web.expasy.org/protparam). The pI of the MNV-1 capsid (NCBI accession number DQ285629.1) has not yet been empirically determined, but it is likely to be similar or slightly lower than the pI of Norwalk virus capsid, since the theoretical pI of the MNV-1 major capsid protein is 4.78 (ProtParam). Considering this pI, when MNV-1 is subjected to SDS in a low-pH solution (pH < 4.0), the net positive charge of the viral capsid may confer attraction to the anionic surface of SDS micelles. Howett et al. proposed that SDS causes viral proteins of nonenveloped viruses to denature and unfold, ultimately leading to inhibition of infectivity (39), a mechanism supported by others (40). At the micelle surface, viral proteins may become destabilized, revealing previously shielded hydrophobic capsid residues. These residues may then be attracted to the hydrophobic tail of SDS, which stabilizes the unfolding of the capsid. Micelle formation and hydrophobic interactions between SDS and proteins have been researched extensively by Otzen et al. (41), and SDS itself has been studied as a virucide effective against both enveloped and nonenveloped viruses (40). The strong wetting property of SDS may have enhanced the efficacy of the sanitizer, enabling it to better moisten the stainless steel surface and thus increase sanitizer contact with the viral particles. Greater contact between sanitizer and virus increases the chance for virus inactivation (42).

Similarly, the kinetic energy (a function of mass and velocity) of spray droplets affects the ability of the sanitizer to dislodge microorganisms from a surface (26). With a hydraulic atomizing pressure of 295 kPa, as used in this study, an average of 0.9 log PFU virus removal was demonstrated with the water and water-plus-SDS liquid controls. In contrast, AAIC electrostatic spraying can be compared to mist spraying in terms of pneumatic atomizing pressure, where the target surface becomes wetted by the sanitizer but the physical force of impact is minimal (43). Consistent with these reported findings, much less virus removal was achieved using the AAIC electrostatic spray nozzle in the current study.

In addition to virus removal, the higher pressure of the hydraulic spray device likely facilitated disruption of the virus-contaminated area by the sanitizers. Theoretical volumes of liquid delivered to the stainless steel surface were calculated and found to be similar for a dual pass using the hydraulic spray nozzle (8.62 μl/cm^2) or six dual passes using AAIC spray nozzle (10.98 μl/cm^2). However, Lyons et al. (28) previously reported that despite delivering the same mass of tracer molecule toward a stainless steel carrier, the deposition rate of a fluorescent tracer molecule onto stainless steel coupons was 9.2-fold greater following AAIC electrostatic spraying than after hydraulic spraying. Less liquid accumulation would therefore result in lower virus-sanitizer contact times and less virus inactivation following hydraulic spraying. But this was not the case. Similar or greater levels of virus inactivation were achieved with hydraulic spraying of 200 ppm chlorine or LEV/SDS, respectively. If the higher pressure of the hydraulic spray helped break up the organic matter of the virus inoculum, this would have resulted in more contact between the virus and sanitizer, but also more contact between the sanitizer and the organic matter (5% FBS) of the virus inoculum. The chlorine sanitizer may have been partially consumed by organic demand of the virus inoculum, which could help to explain its inferiority when used as a hydraulic spray and compared to the LEV/SDS sanitizer, which is effective in the presence of organic matter (21). Park and Sobsey (18) found that free chlorine concentrations of 5,000 ppm only reduced MNV-1, bacteriophage MS2, and FCV by at most 2 log PFU/ml when inoculated onto stainless steel surfaces in the presence of fecal matter. On the other hand, D'Souza and Su (44) reported inactivation of FCV on formica surfaces of 5 logs after a 1-min treatment with 5,000 ppm chlorine when they used a partially purified virus stock. These studies emphasize the variability in chlorine disinfection results for norovirus surrogates due to chlorine consumption by the inoculum matrix and suggest the need for prior cleaning of a surface to remove organic material prior to sanitizing, or the use of alternative sanitizers that are less impacted by organic debris.

Premoistened towelettes proved to be most effective in both virus removal by liquid controls (water and water plus SDS) and in inactivating surface-contaminated viruses. The mechanical action of the wipe application likely helped to dislodge viruses from the surface and to facilitate greater penetration of the sanitizer in the inoculated area. Caution must be advised, however, that sanitizing towelettes be handled with care and that the sanitizer is present at a sufficient concentration on the towelette to inactivate any viruses removed from the surface. Otherwise, viruses may transfer from cleaning cloths to other surfaces that subsequently interface with the cloths, as recently demonstrated by Gibson et al. (12). In our studies, the wipes soaked in sanitizer appeared to be capable of both virus removal and inactivation, but future studies should evaluate levels of virus inactivation on the sanitizer-impregnated towelettes after use to confirm this and assess the risk for surface cross-contamination by used towelettes.

In conclusion, many factors must be carefully considered when deciding upon a sanitizer type and an application method. Each sanitizer and method has its own set of advantages and disadvantages for a particular situation. Factors such as organic load on the surface and evaporative qualities of the sanitizer should be considered when responding with a decontamination procedure. Sanitizers with low evaporative properties and high wetting abilities appear to work well as a wipe, as was shown through the increased log MNV-1 reduction with LEV/SDS and 200 ppm chlorine over Alpet D2. Sanitizers with flammable ingredients should not be used with the AAIC electrostatic spray technology. To maximize sanitizer efficacy against noroviruses, mechanical action plays an important role, as does having a clean surface prior to sanitization. Of note is balancing the pressure of the spray with the efficacy of the sanitizer. Too much pressure without adequate inhibition of pathogen could result in the aerosol dispersal of pathogens throughout the environment, quite the opposite of the desired sanitation effect. Too little pressure may not allow sufficient contact between the virus and sanitizer when used on dirty surfaces. Therefore, high-pressure hydraulic spraying should be conducted in areas where aerosols can be contained or with sufficient sanitizer concentrations to rapidly inactivate pathogens. AAIC

electrostatic spray application methods are best suited for precleaned surfaces where there is no soil to dislodge (43), or when accessing backsides and crevices of otherwise unreachable targets, as shown by Law (37). A recent study by Tuladhar et al. (46) demonstrated improved removal and inactivation of noroviruses by using wipes soaked in 250 ppm chlorine after the surfaces had been wiped once with soap and water, suggesting a two-step cleaning/sanitizing procedure should also be adopted to maximize the effectiveness of sanitizing wipes. Another note of importance revealed by this study was that, while a direct correlation between the removal of the MNV-1 surrogate and removal of human norovirus from surfaces via wiping was difficult to discern, in fact MNV-1 appears to be somewhat more susceptible to degradation by the mechanical action of wiping alone (46). Future studies should consider this potential limitation of the MNV-1 surrogate. In this study, we attempted to control experimental variability by using engineered devices for sanitizer delivery so that the effectiveness of each sanitizer/application method combination could be evaluated. Our findings highlight the importance of evaluating sanitizer/application method combinations to ensure compatibility with one another and suggest that the cleaning of surfaces prior to sanitation will result in greater virus removal and inactivation. Such information is important to consider when developing preventative sanitation or decontamination protocols.

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