SANITIZER EFFICACY AGAINST MURINE NOROVIRUS, A SURROGATE FOR HUMAN NOROVIRUS, ON STAINLESS STEEL SURFACES USING THREE APPLICATION METHODS

by

STEPHANIE LYNN BOLTON

(Under the Direction of Jennifer L. Cannon)

ABSTRACT

Human noroviruses cause over half of foodborne illnesses in the U.S. Since they cannot replicate in laboratory cultures, a surrogate, murine norovirus (MNV-1), is commonly used. This study evaluated three sanitizers, 200 ppm chlorine, levulinic acid plus sodium dodecyl sulfate (LEV/SDS), and isopropanol/quaternary ammonium-based Alpet D2 for efficacy using hydraulic spraying; air-assisted, induction-charged (AAIC) electrostatic spraying; and wiping with an impregnated towelette. Control treatments (water and water/SDS) were included. LEV/SDS proved most effective with hydraulic and AAIC electrostatic spraying, providing viral log reductions of 2.71 and 1.66, respectively. Chlorine and LEV/SDS were equally effective as wipes, reducing viral load by 7.05 logs. Alpet D2 provided a 2.23 log reduction with hydraulic spraying, outperforming chlorine (1.16 log reduction). Controls reduced viral load by < 1 log with spraying applications and by > 3 logs with wiping. Both type and application method should be carefully considered when choosing a food-contact surface sanitizer.

INDEX WORDS: norovirus, murine norovirus, stainless steel, food-contact surface, towelette, air-assisted induction-charged electrostatic spray, sanitize, sanitizer, sanitation, neutralization, elution, levulinic acid, sodium dodecyl sulfate, SDS, SLS, quaternary ammonium compound, isopropanol, isopropyl alcohol, chlorine, wipe, impregnated towelette, saturated towelette

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DEDICATION

I dedicate this work to the families and the friends who have lost or are losing a loved one to norovirus illness. May we all practice better hygiene measures in the future, including proper and frequent hand-washing, and especially those of us working in the food industry.

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CHAPTER 1

INTRODUCTION

Human noroviruses (HuNoV) are a major public health concern in the United States, causing an estimated 5.5 million of the 9.4 million cases of foodborne illness, including nearly 150 deaths, per year *(81)*. Although noroviruses cannot grow outside of a host, they can persist in the environment. Many chemical disinfectants which are effective against bacteria are either completely ineffective against noroviruses or require a stronger concentration and/or longer contact time for inactivating noroviruses. For example, the Alpet D2 sanitizer used in this study inactivates strains of *Staphylococcus*, *Escherichia coli*, *Salmonella*, and *Listeria* by a 5-log reduction in 1 min, but requires a 5-min contact time to reach the same efficacy for noroviruses *(13)*.

Murine norovirus (MNV-1) is a surrogate for human norovirus due to its similarities to HuNoV in terms of genetic organization and structure, and due to its ability to cause enteric infection in mice. This similarity is important since viruses that can survive in the acidic environment of the stomach are likely to be resistant to sanitizers whose inactivation mechanism is dependent on low pH alone. MNV-1 is relatively easy to culture in laboratory tissue using the RAW 264.7 cell line. This study uses plaque assay in RAW 264.7 cells to quantify infectious MNV-1 post-treatment with sanitizers.

The three sanitizers chosen in this study represent three different types of food-contact surface sanitizers that hold promise for the food industry due to their potential power to combat noroviruses. Being various types of sanitizers, they each operate against MNV-1 using different mechanisms of inactivation. LEV/SDS is an acid anionic sanitizer, combining a low pH from the organic acid with the surface-active power of SDS, which likely denatures viral proteins.

The potential for LEV/SDS to become an U.S. Environmental Protection Agency (EPA)-registered and U.S. Food and Drug Administration (FDA)-approved food-contact sanitizer against noroviruses is being evaluated and appears promising. The 200 ppm chlorine sanitizer is widely used in the food industry due to its strong oxidative power. Finally, Alpet D2 is an alcohol-based quaternary ammonium compound sanitizer that is EPA-registered for use against noroviruses, FDA-approved for use on food-contact surfaces, and has the advantage over chlorine in that it is non-corrosive. By comparing sanitizer treatments to control treatments, any added benefit of each sanitizer over tap water and over tap water plus surfactant can be realized, along with any wash-off effect in the case of spray application. Stainless steel was chosen as the food-contact surface in this study due to its widespread use in the food industry.

Application of sanitizer liquids to surfaces by conventional, hydraulic spraying is a traditional means of sanitation, while air-assisted, induction-charged (AAIC) electrostatic spraying, though commercially available, is a more novel technique. AAIC electrostatic spraying shows great promise as a low-volume application method for sanitizers fighting bacterial contamination *(63)*. The use of impregnated towelettes, also known as pre-moistened or saturated wipes, is becoming commonplace as consumers seek quick and easy ways to sanitize. Wipes are ideal for situations where resources such as clean cloths and potable water are limited, for instance farmers' markets or out in the field during harvest.

The objective of this study was to determine how various liquid treatments [the three sanitizer treatments sterile tap water plus 5% levulinic acid (LEV) plus 2% sodium dodecyl sulfate (SDS) surfactant; sterile tap water plus 200 ppm chlorine; and an alcohol-based quaternary ammonium compound (QAC), Alpet D2; and the two control treatments sterile tap water; and sterile tap water plus 2% SDS] compare in their effectiveness at reducing the viral infectivity of a strain of murine norovirus (MNV-1) on solid surfaces using realistic application methods. MNV-1

dried onto stainless steel surfaces was sanitized using three application methods: (1) conventional, hydraulic spraying, (2) AAIC electrostatic spraying, and (3) wiping with an impregnated towelette. Recovery of virus from treated stainless steel coupons was compared to the recovery of virus from recovery controls (untreated coupons inoculated at the same time and in the same manner as treated coupons) in order to determine an average log plaque-forming units (PFU) reduction of infectious MNV-1 for each sample during each replicate. Treatments were compared to each other within each application method, and results will be useful in determining which sanitizer and application method to use for inactivation of noroviruses on stainless steel surfaces.

CHAPTER 2

LITERATURE REVIEW

Human noroviruses (HuNoV), previously called Norwalk-like viruses, were discovered in 1972 by Albert Kapikian following a gastroenteritis outbreak at an elementary school in Norwalk, Ohio *(46)*. Norovirus is the genus name for a group of enteric viruses belonging to the family *Caliciviridae*, which are single-stranded, positive-sense RNA viruses enclosed in a non-enveloped protein capsid. Non-enveloped viruses generally have a greater resistance to treatment with high pressure, heat and drying, as well as to chemical disinfectants. Like many enteric viruses, noroviruses can also survive in low pH conditions as in the intestinal tract (pH range 1.7 to 4), and are therefore resistant to acids.

Noroviruses are small (27-38 nm in diameter) viruses that infect humans and a variety of animals mainly via the fecal-to-oral route. The most common mode of transmission for HuNoV is direct transmission through person-to-person contact *(61)*. There are five genogroups of norovirus (GI-GV), three of which have been detected in humans (GI, GII, and GIV). Atmar and Estes (2006) showed that airborne transmission during vomiting is possible as well, through aerosolized droplets *(7)*. Projectile vomiting can distribute up to 3×10^7 viral particles per 30 mL vomitus into the surrounding environment *(19)*. The virus can also be indirectly transferred through contact with environmental surfaces, or other carriers, due to its environmental stability *(31, 34, 80)*. Water is a noteworthy carrier of norovirus, and the virus has been implicated in outbreaks involving municipal water systems and recreational water *(43, 49)*. Potential for zoonotic transmission of HuNoV has been evidenced in one study by Summa et al. (2012), who demonstrated GII norovirus can survive through a dog's intestinal tract, making

household pets a potential carrier for infection *(85)*. However, further studies are warranted to elucidate the zoonotic potential of noroviruses.

Food is a significant vehicle for norovirus transmission. According to the most recent Centers for Disease Control and Prevention (CDC) estimate, noroviruses account for an estimated 5.5 million of the 9.4 million episodes of foodborne illness per year in the U.S. *(81)*. Typical foods implicated in noroviral gastroenteritis illnesses are shellfish, leafy greens, produce, and ready-to-eat foods *(6, 11, 48, 61, 95)*. Vectors for foodborne contamination include infected food handlers during foodservice operations, harvest workers, food-contact surfaces, and irrigation water *(11, 48, 60)*. A notable outbreak in 1993 occurred after an ill oyster harvester disposed of his feces into an oyster bed - a relatively common practice in the industry *(48)*. Infected food handlers likely cause the greatest number of foodborne cases of norovirus-induced gastroenteritis *(41)*.

It is estimated that in the United States, norovirus causes approximately 23 million episodes of gastroenteritis per year *(68)*. Viral gastroenteritis is defined by Atmar as "illness with moderate diarrhea (alone) for any continuous 24-hour period (moderate diarrhea is > 200 g feces that immediately take the shape of the collection container) or 1 vomiting episode plus 1 of the following: abdominal cramps or pain, nausea, bloating, loose feces (if not fulfilling the definition of watery diarrhea), myalgia, or headache" *(8)*. Usually the illness is self-limiting, but it can cause more severe disease and even death, especially in the elderly and in the immuno-comprised *(21)*. An incubation period of 10-51 h is possible (12-48 h on average), with typical symptoms being vomiting and/or diarrhea lasting 28-60 h, putting people at risk for dehydration *(20, 39)*. Up to 30% of infections may occur without any symptoms *(20)*, which is especially dangerous in terms of spreading the virus because asymptomatic and pre-symptomatic shedding do occur *(11)*. In addition, viral shedding in symptomatic individuals can last up to

eight weeks post-infection *(8)*. HuNoV infectivity is likely unhindered by passing through the human digestive tract and one viral particle has an estimated 49% probability of infection *(88)*.

With a low ID_{50} of as few as 18 viral particles *(88)*, and with one gram of feces potentially containing up to 1,000,000,000,000 (10^{12}) viral particles *(8)*, outbreaks can easily occur if good hygiene practices, especially hand washing, are not followed. Outbreaks are most common in closed-community settings such as schools, residential care homes, hospitals, and cruise ships. In temperate climates HuNoV infections occur most frequently in the wintertime *(59)*. Norovirus is an evolving genus with new strains continuing to appear, making complete immunity to all viral strains likely an impossible feat *(33)*.

Norovirus Contamination in the Environment.

As mentioned previously, many vectors can be involved in the indirect transmission of norovirus between an infected person and a previously-healthy one. With such a low infectious dose, a very small amount of fecal material on unwashed hands is enough to spread the virus to food and beverages or to environmental surfaces, also called fomites *(8)*. Norovirus will not replicate on fomite surfaces since it requires a living host for that function, but can survive and remain infectious on such surfaces for two weeks or more *(60, 84, 87)*. Fomite surfaces, such as stainless steel "prep" tables where ready-to-eat foods are prepared in restaurants, or where heavy human contact is likely, such as bathroom door handles and toilet flushing taps *(34)*, are high-risk vectors and should be cleaned and sanitized properly and frequently. One study showed that a fecally-contaminated fingertip (via pressing the fingertip to contaminated toilet paper for 10 s) can transmit GII norovirus to a series of seven sequentially touched, clean melamine surfaces *(10)*.

Outbreaks where there was no direct human contact between infected parties reveal both the resilience of the infectious virus in the environment and the difficulty in developing

effective control strategies. For example, an ill person infected with norovirus vomited on a large aircraft in 2009. Crew members working eight separate flights following this incident, over a 6-day period, also became ill, even though they were never in physical proximity to an ill person. Genotyping stool samples led to the conclusion that norovirus was transmitted indirectly via the aircraft *(89)*.

Prevention of Norovirus Illness.

The first line of defense for any illness prevention is a healthy immune system, which can be accomplished for most individuals through exercise *(55)*, low stress levels *(79)*, and a healthful diet *(2)*. Good hygiene is crucial in helping to prevent norovirus infection, and includes adequate and frequent hand washing and a clean environment where bathrooms, kitchens, and eating surfaces are regularly cleaned and sanitized *(10)*. Hand washing practices alone can reduce diarrheal episodes by about one-third *(32)*. Unlike bacteria and fungi, whose growth on surfaces typically results in off-odors, slime, and/or discoloration *(90)*, viruses do not grow on surfaces, and therefore easily go undetected by human senses.

Finally, it is important to quarantine ill individuals who work as food handlers or caregivers in homes, schools, cruise ships, and hospitals during the illness and for 2-3 d after symptoms subside *(42)*. Providing paid sick leave for food handlers and the addition of substitute workers to the regular staff may increase the likelihood that ill workers will stay home *(86)*.

Human Norovirus Surrogates for Research.

Efforts have been made to cultivate HuNoV in more than 25 cell lines, but all *in vitro* efforts have been unsuccessful thus far *(30)*. Since human noroviruses cannot yet be cultivated *in vitro*, surrogates are often used for research purposes, especially in disinfection and antiviral studies. Feline calicivirus (FCV) has previously been used as the standard HuNoV surrogate in

sanitation studies and its use is required for making virucidal claims against HuNoV by surface disinfectants, as dictated by the U.S. Environmental Protection Agency (EPA) *(4, 5, 14, 84, 94)*. Unlike norovirus, feline calicivirus causes a respiratory illness and is inactivated at a gastrointestinal pH *(18)*. For many disinfectants, especially those having a low pH, FCV is not the most resistant surrogate available for antiviral testing, and thus is not an ideal choice *(9, 73)*.

In 2003 the first norovirus that could replicate in cell culture, murine norovirus (MNV-1), was discovered *(97)*. Like human norovirus, MNV-1 causes a gastrointestinal illness following ingestion and can survive at a gastric pH *(18)*. Since 2003 several disinfection studies have used MNV-1 as a HuNoV surrogate *(12, 37, 65, 72, 76)*. Furthermore, several side-by-side comparisons of surrogates have found MNV-1 to be more resistant than FCV *(9, 25)*. Another surrogate, coliphage MS2 with an *E. coli* host, is often compared to MNV-1 or FCV in disinfection studies *(9, 73)*. However, like FCV, MS2 shows a similar lower resistance to diluted household bleach than MNV-1 *(25)*. In order to be thorough and to ensure the likeliest outcome for effectiveness, it is wise to combine HuNoV surrogates in research if a laboratory is equipped to do so (with proper cell lines, etc.), and if not, the most resistant surrogate should be used. Since HuNoV is unavailable for laboratory testing, the most resistant surrogate(s) will model the worst case scenario for disinfectant efficacy.

Infectivity Test for Viral Recovery: Plaque Assay.

Several studies have used real-time reverse-transcriptase polymerase chain reaction (RT-PCR) to estimate and quantify viral recovery following a disinfectant application *(12, 31, 72)*. However, RT-PCR does not measure viral infectivity, but rather merely detects viral RNA presence *(31, 73)*. RNA can exist without there being infective viral particles present, due to capsid damage or RNA damage beyond the primer-binding region. In other words, positive RT-PCR results for the presence of viral RNA do not correlate with the presence of infective viral

particles, but rather potentially overestimate the amount of infective virus *(12, 31, 58)*. Plaque assays, on the other hand, can test for remaining viral infectivity using a cell culture. For MNV-1, the cell culture line used is RAW 264.7, and a plaque assay technique was developed by Wobus et al. *(97)*.

Sanitation of Food-Contact Surfaces.

As mentioned, proper sanitation is crucial in preventing norovirus transmission and outbreaks. The first use of the word *sanitizer* was in the *Journal of Milk and Food Technology* in 1950 *(15)*. The Code of Federal Regulations (CFR) defines the word *sanitize* as below:

> " 'Sanitize' means to adequately treat food-contact surfaces by a procedure that is effective in destroying vegetative cells or microorganisms of public health significance, and in substantially reducing numbers of other undesirable microorganisms, but without adversely affecting the product or its safety for the consumer" [21 CFR 110.3 (o)].

In the 2009 Food Code, the Food and Drug Administration (FDA) uses a similar definition for sanitization:

> "'Sanitization' means the application of cumulative heat or chemicals on cleaned food-contact surfaces that, when evaluated for efficacy, is sufficient to yield a reduction of 5 logs, which is equal to a 99.999% reduction, of representative disease microorganisms of public health importance" (FDA Food Code 2009 1-201.10).

Improper sanitation can spread virus to secondary surfaces via the cloth and the hands of the person cleaning *(10)*. Applying an antimicrobial solution alone to a soiled surface is insufficient. A primary cleaning step with a detergent-soaked cloth is often necessary to prepare the surface and to maximize sanitizer effectiveness. This step is vital, since organic matter or other soils can interfere with many chemical disinfectants, as is the case with chlorine-based disinfectants *(10, 66, 73)*. The FDA requires a cleaning step prior to sanitization for food-contact surfaces (FDA Food Code 2009 4-702.11). Research using MNV-1 and stainless steel surfaces supports this instruction, finding that chemical disinfection is improved on clean surfaces versus those with food residues *(87)*.

Choosing the appropriate sanitizer for a particular job is crucial, and should be carefully thought of in the prerequisite program for a HACCP (Hazard Analysis and Critical Control Points) plan. According to the text *Disinfection, Sterilization, and Prevention*, the following features need to be addressed in sanitizer selection: economy (must be cost-effective); ease of application; stability (shelf-life); the production of any harmful or offensive odors; toxicity; cleaning power; penetrative power; residual activity post-application; reduction of vegetative count (ideally by 99.999% in 30 seconds); efficacy before and after dilution with hard water; efficacy in the presence of proteins or organic matter; corrosiveness with respect to the finish of contact surface; broad spectrum of activity toward gram-positive and gram-negative bacteria, fungi, and viruses; fast acting; compatibility with other chemicals and materials of construction; environmental persistence *(15)*. The sanitizer should also be readily available for purchase *(66)*.

Sanitizer: Levulinic Acid plus Sodium Dodecyl Sulfate.

Levulinic acid (an organic acid, $C_5H_8O_3$, structure in Figure 2.1) and sodium dodecyl sulfate (an anionic surfactant that acts as a detergent, *n*-$C_{12}H_{25}OSO_3Na$, structure in Figure 2.1) show promising antimicrobial activity when used in combination, even in the presence of organic matter *(98-100)*.

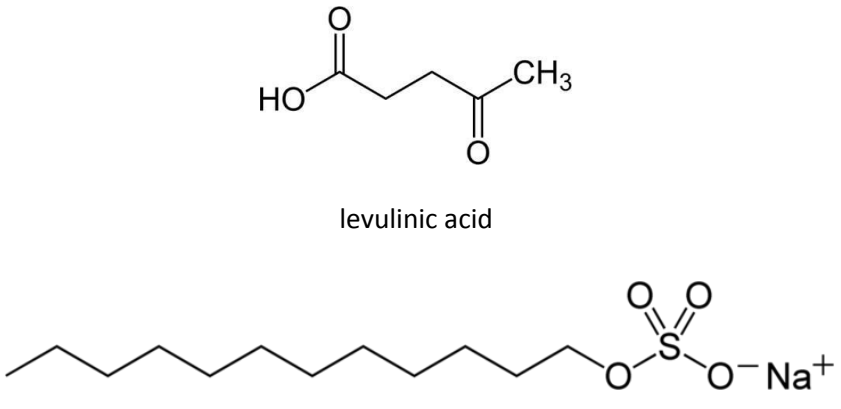

Figure 2.1 The structures of levulinic acid *(45)* **and sodium dodecyl sulfate** *(69)*.

This blend of an organic acid and an anionic surfactant is classified as an acid anionic sanitizer. The characteristics of this type of sanitizer are: stability, low odor, non-staining, effective in a wide temperature range, and unaffected by water hardness *(66)*. Research carried out by The Center for Food Safety at The University of Georgia (Griffin, GA) demonstrated a high reduction of MNV-1 infectivity, using a combination of 5% LEV and 2% SDS in sterile water *(17)*. From this research, it appears that levulinic acid's main role in the sanitizer is that of reducing the pH (unpublished data obtained through personal communication).

Levulinic acid is an FDA-approved food additive (FDA 21 CFR 172.515) and has been used by the cigarette industry to increase nicotine consumption, and therefore addiction, by allowing smoke to reach further into the lungs *(47)*. The conversion of biomass to levulinic acid is currently being studied as levulinic acid has potential use as a "green" platform chemical *(38)*.

SDS is manufactured by the sulfation of long chain alcohols *(90)* and is an FDA-approved food additive under the name sodium lauryl sulfate. It is used as an emulsifier in egg whites, as a whipping agent in marshmallows, and in fruit juices (FDA 21 CFR 172.822). SDS is also an ingredient in laundry detergents, cleaning solutions and pre-moistened wipes, shampoos, soaps, and toothpastes (which often contain concentrations of 1-3% SDS) *(82)*. According to the U.S. Department of Health and Human Services Household Product Database, SDS is an ingredient in hundreds of current and past household products *(91)*.

SDS is also being researched as a potential topical microbiocide to prevent sexually transmitted infections *(74)*. Like other surfactants, SDS lowers the surface tension of a liquid, allowing for greater wetting of a solid carrier. The anionic polar end of SDS is hydrophilic while the nonpolar end is lipophilic; therefore, micelle formation occurs in water-based solvents. Since 5% LEV / 2% SDS has a pH of around 2.7, it creates an environment with a pH below MNV-

1's isoelectric point, and the virus becomes positively charged. Through both hydrophobic and ionic interactions, SDS likely denatures the noroviral capsid proteins *(74)*.

Food-contact surface sanitizers are considered indirect food additives by the FDA (21 CFR 178.1010), and *incidental* food additives under the Federal Food, Drug, and Cosmetic Act (as amended 21 U.S.C. 201 et seq.). Since both levulinic acid and sodium dodecyl sulfate are approved as food additives, there should be neither toxicity nor any chemical safety issues dissuading the approval of LEV/SDS as a surface disinfectant for use on food-contact surfaces. However, evaluation is warranted.

Sanitizer: 200 ppm Chlorine.

Chlorine-based solutions are in widespread use as no-rinse sanitizers for food-contact surfaces *(15)* and as such may contain up to 200 ppm chlorine as approved by the FDA (21 CFR 178.1010 (b)(1), 21 CFR Part 178.1010 (c)(1)). In fact, U.S. EPA testing requires concentrations from 50-200 ppm hypochlorite to be used as the standard for comparison in their food-contact sanitizing rinse approval process *(93)*. As early as 1929, chlorine was studied and recommended for its sanitation purposes, starting with dairy farm and milk plant equipment *(77)*. Small test strips, indicating the ppm level of available chlorine in a given solution, are inexpensive to purchase and are required by some county health departments to be present in foodservice establishments where chlorine is in use *(personal communication with foodservice employees)*.

Despite the widespread use of chlorine bleach as a sanitizer, there are several disadvantages to its use. Chlorine's strength as an oxidizing agent makes it a powerful tool against microbes, but it also causes the corrosion of metal surfaces. Also, organic matter, such as food debris, can play a protective role for pathogens, decreasing the strength of chlorine as a sanitizer *(90)*. The water used to make the chlorine solution cannot be too hard or too alkaline, and must be at a specific minimum temperature based on the desired concentration and the pH

[FDA Food Code 2009 4-501.114(A)] to ensure presence of free chlorine, particularly hypochlorous acid (HOCl), which is the more potent form of the sanitizer (Figure 2.2).

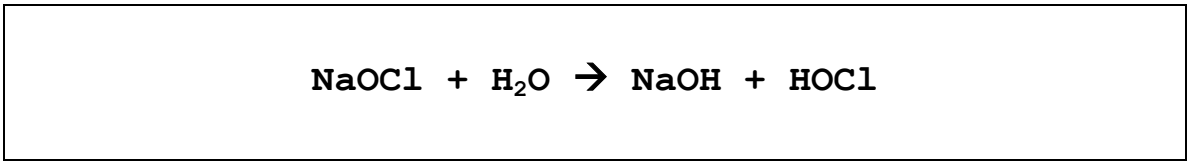

Figure 2.2 Chemical equation for the production of HOCl in aqueous solution *(66)***.** Sodium hypochlorite plus water yields sodium hydroxide plus hypochlorous acid.

Solutions of chlorine are unstable over long periods of time, weakening by dissipation upon exposure to ultraviolet radiation and air or by combining with metals and organic materials. This instability arises due to chlorine's high electronegativity and affinity for electrons, which causes it to be a strong oxidizing agent, likely to become reduced. Once opened, stock bleach containers (5-6.25% sodium hypochlorite) should be used within 30 days *(15)*.

Park and Sobsey (2011) found that free chlorine concentrations of 500 ppm only reduced MNV-1, MS2, and FCV by at most 2 log PFU/mL when inoculated onto stainless steel surfaces in the presence of fecal matter. These researchers concluded that a primary washing step with a detergent is necessary to first reduce viral load on a surface prior to disinfection with chlorine *(73)*.

Sanitizer: Alpet D2.

Best Sanitizers, Inc., (Penn Valley, CA) produces a sanitizer/disinfectant called "Alpet D2" that is EPA-registered as a surface disinfectant, effective against norovirus, HIV-1, Hepatitis B, and a variety of pathogenic bacteria, and FDA-approved for use on food-contact surfaces *(94)*. Alpet D2 is an isopropyl alcohol-based (58.6%) quaternary ammonium (octyl decyl dimethyl ammonium chloride, dodecyl dimethyl ammonium chloride, and dioctyl dimethyl ammonium chloride) solution, claiming a 5-log reduction against bacteria and viruses when used as directed. Quaternary ammonium compounds generally are ineffective at inactivating non-enveloped,

enteric viruses, such as noroviruses *(37)*. Also, solutions of 70% ethanol *(31)* and up to 60% isopropyl alcohol demonstrate limited efficacy against noroviruses inoculated onto stainless steel *(65)*. For Alpet D2, a 5-min contact time is required for disinfection of norovirus on nonporous surfaces and manufacturer instructions include a pre-cleaning step of the surface to be sanitized. A coarse spray is also recommended for spray applications. There is no required rinse step after sanitation (E.P.A. Reg #73232-1), and since Alpet D2 is highly evaporative, non-corrosive, and non-conductive, it can be used as a surface sanitizer in the presence of and directly onto electrical connectors and conductors inside electric and electronic control panels.

Food-Contact Surface: Stainless Steel.

According to FDA's 2009 Food Code, a "'food-contact surface' means: (1) A surface of equipment or a utensil with which food normally comes into contact; or (2) A surface of equipment or a utensil from which food may drain, drip, or splash: (a) Into a food, or (b) Onto a surface normally in contact with food." (FDA Food Code 2009 1-201.10). Stainless steel is commonly used as a food-contact surface in kitchens, foodservice establishments, and food processing equipment and facilities *(90)*. Stainless steel is less corrosive than other metals, easier to rid of microbes than polymers or aluminum *(16)*, and is durable. Specifically, Type 304 Finish #4 stainless steel, an 18-8 grade of steel made of ferrous metals plus nickel and/or chromium as the main alloy components, is used in kitchens and in foodservice establishments *(90)*. It is suspected that norovirus can survive on a clean stainless steel surface for nearly 30 days or on a stainless steel surface with food residue for longer (MNV-1 was used as a surrogate, initial inoculum = 6.4 log MPN/mL) *(87)*. This resilience of norovirus stresses yet again the importance of a cleaning step to remove soil, or "matter out of place," prior to sanitizing *(90)*.

Application of Sanitizers in the Food Industry.

According to the FDA Food Code, which is currently adopted by 49 states, EPA-registered antimicrobials may be applied to food-contact surfaces by "immersion, manual swabbing, brushing, or pressure spraying methods" (FDA Food Code 2009 4-703.11(C)). It is always important to follow manufacturer instructions when applying any sanitizer, which may include any or all of these application methods. There are hundreds of sanitizers on the market to be used as rinses, sprays, and immersion solutions. Efficient sanitization methods for pathogenic microorganisms involve a three step process: a thorough cleaning of the surface to remove organic debris *(87)*, mechanical action such as scrubbing to disrupt microbe attachment, and application of a sanitizer following manufacturer instructions to significantly reduce the pathogenic load *(15)*.

The text *Hygiene in Food Processing* claims that four major factors can contribute to sanitation: mechanical or kinetic energy, chemical energy, temperature or thermal energy, and time. Specifically, mechanical energy is necessary to physically dislodge soils, and methods include scraping, scrubbing, brushing, and fluid abrasion *(57)*. Chemical energy is necessary to attack the pathogens on a molecular level, disrupting membranes and vital functions through intermolecular forces and/or the formation of micelles. Thermal energy can damage microbes of public health concern, depending on the thermal tolerance of the pathogen, and/or can enhance chemical energy, as with chlorine. Air-drying post-sanitation is also recommended to keep the surface free of pathogens *(1)*. Most sanitizers increase effectiveness as the contact time increases, up to a certain point. However, shorter contact times are desirable for time efficiency and cost-effectiveness.

When using a chemical sanitizer concentrate that is to be diluted with water for use, it is important to measure the pH and the hardness of the water. For some sanitizers, an increase in

water hardness (over 200 ppm) decreases effectiveness, because the added ions in the water (Ca^{+2}, Mg^{+2}) may react with the chemicals in the sanitizer, or may bind to the surface of the target microbe and block the chemical action *(66)*. According to the U.S. Department of the Interior, hard water has between 121-180 ppm, and is more likely to occur in the Midwestern United States than in other regions of the country *(92)*. Interestingly, the proposed Organisation for Economic and Co-Operative Development Draft "Guidelines for the Testing of Chemicals," written for its 34 member countries, requires the use of hard water in virucidal efficacy testing *(70)*. Similarly, the state of Wisconsin requires that food-contact surface sanitizers must remain effective in water which has up to 500 ppm hardness *(96)*.

Application Method: Hydraulic Spray.

As there are a wide range of available spray sanitizers marketed for use in homes and in foodservice operations, spray-based application methods appear to be common. Usually, the target surface is pre-cleaned, the sanitizer is sprayed onto the surface, and then the sanitizer is wiped with a clean reusable cloth, a disposable cloth, or a paper towel after the desired contact time has elapsed. If the sanitizer is a no-rinse food-contact surface sanitizer, then the surface is ready for use. However, some sanitizers require a rinse step and/or air-drying following use.

Spray bottles or other hydraulic-atomizing (meaning a liquid forced by hydraulic pressure through a confined space or nozzle orifice to form spray droplets) apparatuses may be used to apply sanitizers. Droplet size and projection force vary with the spraying system. Such hydraulic sprays typically have spectrum of sizes and a median diameter of approximately 300 µm on a mass or a volume basis *(52, 63)*. As mentioned previously, energy is an important factor in sanitation success. The kinetic energy (a function of mass and velocity) of spray droplets affects the ability of the sanitizer to dislodge microorganisms, with increasing pressure causing an increase in kinetic energy *(75)*. Of important 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.

Several types of spraying for cleaning and sanitation purposes exist in the food industry. For example, conventional spraying systems are used in the fresh produce industry to wash and sanitize post-harvest fruits and vegetables *(22)*. Chang and Schneider (2012) found that 100 ppm chlorine was more effective against *Salmonella* when applied to tomatoes as an overhead spray than applied in a flume immersion setting *(22)*. As another example, a grocery store chain in the Cayman Islands uses a fine-mist spraying system to sanitize grocery carts during flu season *(3)*.

Application Method: Air-Assisted, Induction-Charged Electrostatic Spray.

The application of liquid treatments such as sanitizers, disinfectants, and pesticides can be further managed and controlled through the use of air-assisted, induction-charged (AAIC) electrostatic spray technology. In the late 1970s at The University of Georgia, Law developed a pneumatic atomizing, electrostatic-induction charging nozzle for the efficient production and air-assisted delivery of highly charged droplets of spray liquid (with a low-to-middle electric resistivity range of between 10^{-1} to 10^{4} ohm m typical of common tap water) onto agricultural plants *(50)*. Law evaluated the principles of ionized-field particle charging and contact charging before selecting the electrostatic-induction charging principle as optimal for safe and reliable nozzle design and creation for the agricultural and food processing sectors *(50)*. Advantages of this nozzle type include a compact size, simplicity, low cost, and compatibility with conventional spraying systems *(50)*. Within the nozzle, a high-velocity air flow coaxially interacts with the liquid flow, thus providing enough kinetic energy to atomize, or shear, the liquid into approximately 30 µm-diameter droplets *(50)*. The induction electrode within the nozzle is set

close enough to the grounded droplet-formation zone so that moderately low electrode applied voltage is able to induce a significant charge on the droplets (-12 to -15 μA) at their instant of formation, enough to overcome gravitational forces to coat the desired target surface *(50)*. Also, the charge-inducing electrode is embedded within the insulating nozzle, which with the high velocity air flow stays completely dry – increasing safety and minimizing personal injury, misalignment, and damage to the equipment *(50)*.

Today, The University of Georgia Research Foundation licenses its patents on AAIC electrostatic spray technology for commercialization worldwide (www.electrostaticspraying.com). In addition to agriculture, AAIC electrostatic spraying is used in several other industries, such as the wine industry (for pesticide application on grapevines) *(personal communication with an anonymous winery)* and the sunless tanning industry (facilitating better, more even coverage of tanning solutions) *(24)*. Liquid treatments applied to a target surface with AAIC electrostatic spraying systems use a low volume of liquid to achieve a desirable coating, and a wrap-around effect of liquid particles allows coverage of hidden areas such as under- or back-sides, as occurs with plant foliage *(27, 53)*. At a -7.2 mC/kg average droplet charge-to-mass ratio imparted to the spray, Lyons found that AAIC electrostatic spraying increased the active ingredient deposition on the backside of a target surface 29-fold as compared with a conventional, hydraulic spray application method *(63, 64)*. In-field studies with cotton and vegetable crops showed that half the amount of pesticide active ingredient, dispensed in greatly reduced volumes of spray-mix liquid, was needed using AAIC electrostatic application as opposed to conventional applications to obtain the same effectiveness of insect control *(51, 52)*. Furthermore, Ganesh et al. (2010) showed that solutions of food-grade acids and a food-grade acid with grape seed extract applied to spinach using AAIC electrostatic spray were more effective against a strain of *Salmonella* than if applied using conventional spray *(35)*.

Application Method: Saturated Wipes.

While antibacterial wipes exist and are in widespread use today, antinoroviral wipes are currently in the research phase. Some sanitizer formulations, like Alpet D2, are EPA-registered as a surface disinfectant against noroviruses in their liquid form with a 5-min contact time (based on FCV testing), but as a saturated towelette, they are only registered for use against bacteria. Clorox brand makes a disinfecting wipe that claims to kill 99.9% of rhinoviruses and Influenza A2, but requires enough impregnated wipes 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 *(23)*.

Wipes, which provide not only the impregnated sanitizer but also inherently use frictional force during application, are advantageous because they are ready and easy to use. Sanitizing properly requires persistence and energy, and methods to make the task easier likely increase the probability that it occurs. Also, wipes are disposable, thus when they are used on one surface at a time, the potential for pathogen spreading and cross-contamination declines. Furthermore, for farmers' markets, hot dog stands, and in-field packing operations, or anywhere else where access to potable water is limited, no-rinse food-contact surface wipes would be ideal for sanitizing on-the-go.

CHAPTER 3

MATERIALS AND METHODS

Virus Cultivation and Plaque Assay.

RAW 264.7 macrophage cells from *Mus musculus* (TIB-71, ATCC, Manassas, VA) were maintained and grown in complete DMEM [Dulbecco's modified eagles medium (Invitrogen, Grand Island, NY) containing 10% low endotoxin fetal bovine serum (HyClone FBS (#SH3007103; Thermo Fisher Scientific, Inc., Logan, UT), 1% penicillin/streptomycin, 1% HEPES buffer, and 1% sodium pyruvate (Invitrogen, Grand Island, NY)], and passaged every 2-3 d. Confluent monolayers (80-90%) of cells maintained in complete DMEM were infected with a partially purified cell culture lysate of MNV-1 for approximately 48 h at 37°C in a 5% CO_2 environment.

Virus was harvested via three cycles of freeze-thawing when complete cytopathic effect was apparent. A partially-purified cell culture lysate of MNV-1 was purified via centrifugation at 2,000 x *g* for 15 min at 20°C and filtered using a 0.2 µm membrane filter (Nalgene, Rochester, NY). For all experiments described herein, the lysate was concentrated via ultracentrifugation at 85,000 x *g* for 1 h at 4°C and the pellet was re-suspended overnight in 1/10 the original volume in phosphate buffered saline (PBS; 8 g/L NaCl, 0.2 g/L KCl, 0.12 g/L KH_2PO_4, 0.91 g/L Na_2HPO_4 with pH adjusted to 7.5) containing 5% FBS (v/v). One mL portions of MNV-1 were stored in sterile centrifuge tubes (1.5 mL, VWR, Radnor, PA) and frozen to -70 ±2°C (Innova, U535, Ultra Low Temperature Freezer, New Brunswick Scientific, Edison, NJ) until used.

Standard plaque assay was performed to quantify viral infectivity for treatment and control samples (for protocol, see Appendix E). Cells were grown to 80-90% confluence on 60 mm x 15 mm tissue culture plates (#353004, BD Falcon, Franklin Lakes, NJ) containing 5 mL

complete DMEM. Media was replaced with 400 µL of 1x modified eagle's medium (MEM; 1% 1 M HEPES buffer, 1% penicillin and streptomycin, 1% 100 mM sodium pyruvate, 5% HyClone FBS and 42% MEM) and 100 µL of undiluted or serially diluted (in 1x MEM) samples. Samples were plated in duplicate. Plates were incubated for 1 h at 37°C with 5% CO_2, while rocking trays of cells every 15 min, to allow virus adsorption to cells. At the end of the hour, 1x MEM and the plated sample were removed from cells via aspiration, and cells were overlaid with 3 mL of 1x 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. Duplicate negative controls with and without agarose and duplicate positive controls with and without agarose, and including a low dilution of virus, were included to ensure that virus infection could be visualized on positive control plates and that the negative control cells remained healthy (for a more detailed description of controls, see Appendix F).

Following the 48 h incubation period, a second agarose overlay consisting of 3 mL of 0.75% agarose with 1.1% neutral red (0.005 g/mL, Sigma-Aldrich, Co. LLC, St. Louis, MO) was administered to each plate. After 4-8 h, plaque-forming units (PFUs) were counted and used to determine the titer of virus. Log reduction in MNV-1 PFU was calculated by subtracting the log PFU MNV-1/mL recovered from treated coupons from the log PFU MNV-1/mL recovered from the average of the five recovery controls included in each replicate (see Plate Recovery Controls in Appendix F).

Virus Inoculation onto Stainless Steel Surfaces.

Stainless steel coupons (5 cm x 2 cm x 1 mm, Type 304, Finish #4, UGA Research Services Instrument Shop, Athens, GA) 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 in a new container, dried with wipes (Kimwipes, Kimberly-Clark, Neenah, WI), autoclaved at 121°C for 30 min and stored in a sterile container until used.

Sterile stainless steel coupons were spot-inoculated with five 15-µl portions (75 µL total volume) of MNV-1 virus stock or five 15-µl portions (75 µL) of PBS containing 5% FBS (v/v) (previously frozen to -20°C), serving as a negative control. The inoculum was then evenly spread over each coupon in a pattern (Figure 3.1) using a pipet tip to create a thin liquid surface layer.

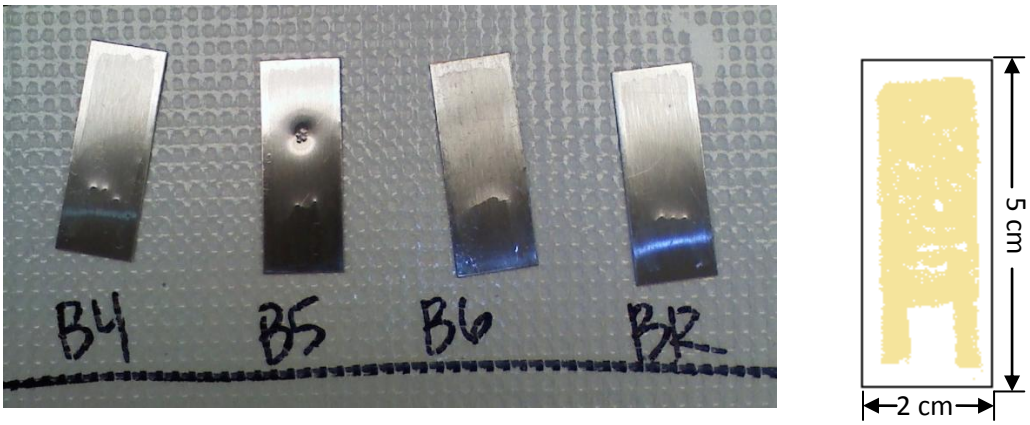

Figure 3.1 Stainless steel coupons inoculated with MNV-1: a photograph of actual coupons (left) and a diagram (right). This figure demonstrates how the inoculation pattern is such that an alligator clip can be used to hold the coupon at the bottom center without touching any inoculum.

Coupons were allowed to dry on the bench top 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. A sample recording log for coupon inoculation and treatment time sheet, which was used for the second wipe application replicate, is included in Appendix A.

Five inoculated coupons per experimental replicate were included as no treatment, recovery controls. Relative humidity was measured during drying and varied from 46 to 63% between replicates for the conventional hydraulic spray application (Table 3.2), from 35 to 60% between replicates for the AAIC electrostatic spray application (Table 3.3), and from 26 to 53%

between replicates for the wipe application (Table 3.4). For all replicates, the temperature range was within 20.5 ± 1.3°C.

Table 3.2 Ambient conditions for drying MNV-1 on stainless steel coupons before replicates of hydraulic spray application.

HYDRAULIC	DRYING TIME	RELATIVE HUMIDITY	TEMPERATURE
REP 1	70 min	63%	19.5-19.6°C
REP 2	70 min	54-56%	19.7-20.5°C
REP 3	70 min	46-47%	20.9-21.8°C

Table 3.3 Ambient conditions for drying MNV-1 on stainless steel coupons before replicates of AAIC electrostatic spray application.

ESS	DRYING TIME	RELATIVE HUMIDITY	TEMPERATURE
REP 1	110-185 min	60%	19.6°C
REP 2	100-115 min	57%	20.0-20.3°C
REP 3	60-80 min	35-39%	19.7-20.0°C

Table 3.4 Ambient conditions for drying MNV-1 on stainless steel coupons before replicates of wipe application.

WIPE	DRYING TIME	RELATIVE HUMIDITY	TEMPERATURE
REP 1	50 min	26%	19.3-19.9°C
REP 2	75-85 min	52-53%	20.5-20.9°C
REP 3	65-85 min	47-48%	21.2-21.3°C

Preparation of Sanitizers and Controls.

Tap water from one single laboratory source was sterilized by autoclaving at 121°C for 15-30 min, depending on volume of water. Solutions were prepared on the day of use in sterile glass bottles for sterile tap water plus 2% SDS (w/v) (Sigma-Aldrich Co., St. Louis, MO), sterile tap water plus 5% levulinic acid (LEV; Sigma-Aldrich Co., St. Louis, MO) plus 2% SDS (5% LEV / 2% SDS), and 200 ppm chlorine solutions [prepared from household bleach (6.25% sodium hypochlorite), Inter-American Products, Inc., Cincinnati, OH]. Free, available chlorine was measured using a chlorine titration (iodometric, starch-iodide method) kit (Chlorine Test Kit #101, Ecolab Center, St. Paul, MN) to ensure 200 ppm free chlorine (kit measurements were

accurate in 10 ppm increments). Alpet D2 was purchased from Best Sanitizers, Inc. (Penn Valley, CA) and used without dilution, per manufacturer instructions.

Measurements of pH were taken for the sterile tap water, sterile tap water plus 2% SDS, 5% LEV / 2% SDS, and 200 ppm chlorine solutions for each experimental replicate (except for one day of the hydraulic application, where only pH for sterile tap water was measured). Hardness was measured for the sterile tap water using a water hardness kit (#402, Ecolab Center, St. Paul, MN) and values ranged from 34 to 68 ppm throughout all experimental replicates, indicating that the water used was not hard water. Electrical conductivity of spray liquid for all treatments was measured for the AAIC electrostatic spray (ESS) application only, using a conductivity meter (HI 8733, Hanna Instruments, Ann Arbor, MI). Values of pH, water hardness (wh), and electrical conductivity (c) for each replicate of each application method are listed in Tables 3.5-3.7.

Table 3.5 Values of pH and water hardness (wh) for hydraulic spray replicates.

HYDRAULIC	WATER	2% SDS	5% LEV / 2% SDS	200 ppm CHLORINE	ALPET D2*
REP 1	9.21, wh = 34 ppm	ND	ND	ND	6.93
REP 2	9.03, wh = 43 ppm	ND	2.75	8.80	6.93
REP 3	7.76, wh = 68 ppm	8.02	2.79	8.95	6.93

*Alpet D2 was used undiluted; all batches were from the same lot with the same expiration date, and thus the pH was measured only one time.
ND = Not Determined

Table 3.6 Values of pH, water hardness (wh), and electrical conductivity (c) for AAIC electrostatic spray replicates.

ESS	WATER	2% SDS	5% LEV / 2% SDS	200 ppm CHLORINE	ALPET D2*
REP 1	7.51, wh = 51 ppm, c = 858 µS/cm	6.20, c = 2280 µS/cm	2.67, c = 3710 µS/cm	8.93, c = 1015 µS/cm	6.93, c = 10.3 µS/cm
REP 2	8.17, wh = 51 ppm, c = 146 µS/cm	8.31, c = 2300 µS/cm	2.67, c = 3650 µS/cm	9.06, c = 1420 µS/cm	6.93, c = 10.0 µS/cm
REP 3	8.51, wh = 51 ppm, c = 145 µS/cm	9.51, c = 2320 µS/cm	2.70, c = 3710 µS/cm	9.16, c = 981 µS/cm	6.93, c = 10.3 µS/cm

Table 3.7 Values of pH and water hardness (wh) for wipe replicates.

WIPE	WATER	2% SDS	5% LEV / 2% SDS	200 ppm CHLORINE	ALPET D2*
REP 1	7.48, wh = 42 ppm	8.24	2.72	8.88	6.93
REP 2	8.37, wh = 68 ppm	5.92	2.61	9.06	6.93
REP 3	9.09, wh = 59 ppm	8.86	2.58	8.80	6.93

Optimization of Neutralization and Elution Buffers for this Study.

Due to the different types of sanitizing treatments (LEV/SDS, 200 ppm chlorine, and Alpet D2) used in this study, it was necessary to optimize the neutralization procedure. Also essential was determining the best elution technique for removal of virus from stainless steel coupons. These preliminary experiments were conducted with the following neutralization solutions: DE broth (Dey-Engley neutralizing broth, Sigma-Aldrich, Co. LLC, St. Louis, MO), DE broth plus sodium bicarbonate (3.7 g sodium bicarbonate/1 L DE broth), letheen broth (letheen broth base with supplemented tween 80, Neogen, Lansing, MI), complete DMEM (HyClone, high glucose, with 15.9 mg/L phenol red, #SH30022.02, Thermo Fisher Scientific, Inc., Logan, UT), complete DMEM plus sodium thiosulfate (0.01 g sodium thiosulfate/300 mL DMEM), and a novel "neut/elut combo" solution [letheen broth with tween 80 (Neogen Corporation, Lansing, MI) plus 1M NaCl, 0.01 g sodium thiosulfate/500 mL, and 2.0 g sodium bicarbonate/500 mL]

(Table 3.8). In the case of letheen broth, a lesser amount of neutralizer solution (10 mL vs. 15 mL) was also tested for efficacy with Alpet D2.

Table 3.8 Sanitizer and neutralizer combinations used to optimize a neutralization procedure for all three sanitizers.

Sanitizer Type	Neutralizer Solution	Amount of Neutralizer (mL)
Alpet D2	neut/elut combo	15
Alpet D2	Letheen	15
Alpet D2	DE broth	15
Alpet D2	Letheen	10
200 ppm Chlorine	neut/elut combo	15
200 ppm Chlorine	DMEM + sodium thiosulfate	15
200 ppm Chlorine	DE broth	15
LEV/SDS	neut/elut combo	15
LEV/SDS	DMEM	15
LEV/SDS	DE + sodium bicarbonate	15

To test elution buffers, an experimental comparison between the neut/elut combo and 0.1 M high salt PBS with tween 20 [sterile Milli-Q water with 1 M NaCl, 3 mM KCl, 0.882 mM KH_2PO_4, and 6.4 mM Na_2HPO_4, 0.5% tween 20 (Sigma-Aldrich, Co. LLC, St. Louis, MO), pH 7.2], a common elution buffer for virus recovery from stainless steel surfaces, was performed.

Uninoculated stainless steel coupons were submerged in the appropriate sanitizer solution for a total of 5 min, followed by a 1 min air-drying time. Then, the coupons were immediately placed into the appropriate 50 mL tube (#430828, Corning, Inc., Corning, NY) containing neutralizer solution. Tubes containing coupons were gently inverted three times, allowing neutralizer solution to completely cover the coupon each time. In the case of the neut/elut combo, the tube was also vortexed at full speed for 30 s (Vortex-Genie 2, VWR, Radnor, PA). Coupons were removed with sterile forceps. A 75 µL portion of each solution was removed and replaced with 75 µL of a ten-fold dilution of MNV-1. Tubes were vortexed and a 1 mL aliquot of each sample was frozen to -80 ±2°C for at least 4 h, then assayed. Two samples

per test neutralizer were included and all results were obtained in one single plaque assay with appropriate controls.

To determine the efficiency of elution, six stainless steel coupons were inoculated with 75 µL each of a tenfold dilution of MNV-1. Post-inoculation, coupons were allowed to dry on a bench top in a biosafety level 2 laboratory room. Once dry, they were placed in the appropriate elution solution (three coupons in neut/elut combo and three coupons in high salt PBS) in a 50 mL tube, inverted three times, then vortexed for 30 s at full speed. Coupons were removed using sterile forceps (#Z168777, Sigma-Aldrich, Co. LLC, St. Louis, MO) and a 1 mL aliquot was taken and frozen to -80 ±2°C for at least 4 h before being tested by plaque assay.

Virus Elution from Stainless Steel Coupons.

Post-treatment, each stainless steel coupon plus one recovery control was immediately placed in a sterile 50 mL centrifuge tube (#430828, Corning, Inc., Corning, NY) containing 15 mL of neut/elut combo at room temperature. Each tube was gently inverted three times to allow the liquid to completely contact the surface of the coupon before vortexing at full speed for 30 s (Vortex-Genie 2, VWR, Radnor, PA). Coupons remained in the neut/elut combo post-rocking (up to 1 h 45 min duration of time) but were removed using sterile forceps (#Z168777, Sigma-Aldrich, Co. LLC, St. Louis, MO) before samples were aliquoted into two 500 µL portions for storage at -80 ±2°C for at least 4 h before plaque assay. The remaining (~13 mL) liquid from each sample for the wipe application method was also stored at -80 ±2 °C.

MNV-1 Concentrated by Polyethylene Glycol (PEG) Precipitation.

For 19 samples (of the wipe application experiments) where no infectious virus could be detected on the zero dilution plates, a PEG precipitation method was used to lower the assay limit of detection. In this procedure, 8% (w/w) polyethylene glycol (PEG 8000; Sigma-Aldrich, Co. LLC, St. Louis, MO), was added to each sample in 50 mL centrifuge tubes, which were then

immediately vortexed for approximately 5 s. Tubes were placed in a 4°C incubator fitted with a shaking rack overnight. The following day, samples were centrifuged at 9,000 x *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 high salt PBS with vortexing. Samples were portioned into two 500 µL aliquots and frozen to -80 ± 2°C for at least 4 h before assaying.

Conventional, Hydraulic Spray Application.

All spray applications were implemented utilizing a University of Georgia laboratory facility developed and fully described previously *(63, 64)*; it is briefly summarized as follows. Inside a biosafety level 2 laboratory room, a stainless steel smokehouse oven (Alkar model 450, Alkar-RapidPak, Inc., Lodi, WI) was modified into a robotically-controlled spray chamber. The smokehouse door was replaced with a plastic dam 2.54 cm in height at the bottom, to contain liquid run-off, and 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 air flow, and remained in the "on" position during all replicates. The electrically-grounded spray chamber was 1.5 m x 1 m x 1 m, and was fitted with a tall ring stand holding a metal arc at a height of 83 cm which allows coupons to attach radially using sterilized alligator clips (Ideal Industries, Inc., Sycamore, IL) (Figure 3.9).

Figure 3.9 Front view of the spray chamber converted from a smokehouse oven.

The target-holding metal arc had four equidistant holes spaced 15.24 cm apart, with thumb screws to affix the alligator clips. This set-up 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 treatment for each replicate. 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 prescribed distance between the spraying nozzle tip and the target coupon was 42 cm.

The hydraulic spraying apparatus consisted of a liquid-holding reservoir, a pump and motor, a by-pass 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 treatment solution was contained in an 8-L plastic reservoir (Nalgene, Rochester, NY) with an on/off valve, which was connected to a twin piston pump (Dayton model 6AWC3, USA), to deliver a nozzle

pressure of 43 psig as measured by a gauge (Weksler, Deer Park, NY) with excess by-passed liquid returned to the reservoir (Figure 3.10).

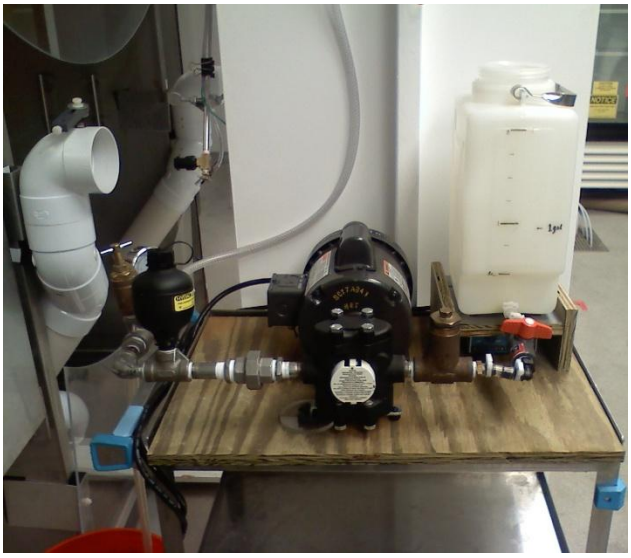

Figure 3.10 Close-up view of the hydraulic reservoir and pump system.

Treatment solution flowed through plastic tubing into a 0.635 cm diameter stainless steel, tubular robotic arm 0.99 m in length. Attached perpendicular to the end of the 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 (VMD) 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 x 10.2 cm thick vertical flat fan spray pattern, which passed over the coupons from left-to-right and from right-to-left in one dual-pass sweep with a 120° spray arc (in sync with the metal coupon-holding arc), lasting 6,000 ± 200 ms. An electronic controller and digital timer facilitated accurate and repeatable pass-time of the robotic arm mounted nozzle.

One L of each treatment solution was allowed to flush through the set-up before being applied to the coupons, and treatment solutions were continuously stirred during application using a magnetic stir bar and a stir plate (#58940-158, VWR, Radnor, PA) underneath the

reservoir. A sterile tap water rinse of at least 2 L 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, and LEV/SDS treatments, because they 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 time allowance in the chamber for air-drying before being placed into the neut/elut combo solution.

Step-by-step instructions for using the hydraulic spray apparatus are in Appendix B.

AAIC Electrostatic Spray Application.

The same spray chamber and electronic-controlled robotic arm as the hydraulic application was used for the air-assisted, induction-charged (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 *(50)* 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 µm. Compressed air, controlled by a valve and measured with a pressure gauge (Weksler, Deer Park, NY) flowed through the steel robotic arm and atomized the spray liquid. The treatment solution flowed 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) which 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 (0-12 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 -12 µA onto the conductive liquid of the water-based spray cloud (except for the non-

conductive, alcohol-based Alpet D2, which only reached a charge flow of approximately -5.2 µA). A digital multimeter fitted with a 26-gauge needle ionization probe, held on the center line of the spray cloud at 2-3 cm from the nozzle face (Model 410, Extech, Waltham, MA), measured and verified the charge convected on the spray (Figure 3.11).

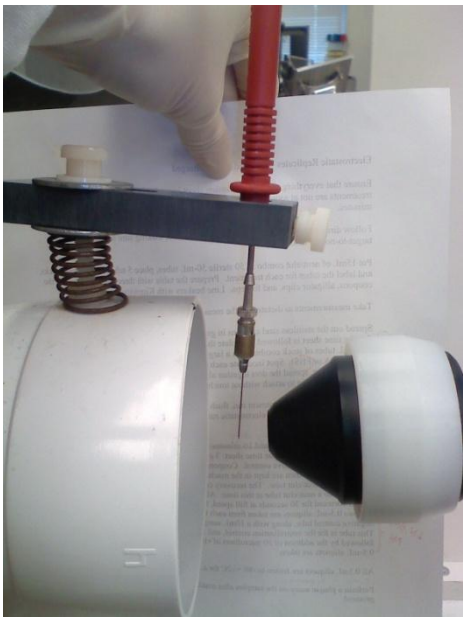

Figure 3.11 Close-up view of the probe and the AAIC ESS nozzle.

From the time rates of charge conveyed on the spray cloud and the liquid flow, which was measured and stabilized at 100 mL/min 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 -3.75 mC/kg). For calculations of the average charge-to-mass ratio for the spray cloud, see Appendix G.

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. Also, since the treatment flow rate for the 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.

Previous spray-sanitization studies conducted in this biosafety laboratory spray facility by Lyons et al. (2011) have established operational conditions which help to ensure unbiased experimental comparisons (based both upon efficiency of mass deposition and microbiocidal efficacy) of various spray-application methods *(63, 64)*. The guiding constraint was to dispense an equal mass of sanitizer active ingredient (A.I.) toward each unit area of the target coupon facing the spray vector. Using an accurately timed dual-pass by the target, each method of application was required to have developed the same width of spray swath (30.5 cm) at the target and the product of spray-mix liquid flow rate (mL/min) multiplied by the concentration of active ingredient in the spray-mix would be equal. Namely, for conventional hydraulic spray application vs. low-volume AAIC electrostatic spray application, this required, respectively, (600 mL/min x 1-X concentration A.I.) vs. (100 mL/min x 6-X concentration A.I.). In this experiment, equalization of the mass of sanitizer applied for hydraulic spray and AAIC electrostatic spray was attempted by using one dual-pass sweep for the hydraulic spray application at a 600 mL/min flow rate, and six consecutive dual-pass sweeps for the AAIC electrostatic spray application at a 100 mL/min flow rate. Thus, no concentrations of active ingredients for sanitizer solutions were altered from their desired formulations, and no additional wash-off effect due to higher dilutions with water could inhibit an accurate portrayal of treatment efficacy.

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/elut combo solution.

A step-by-step protocol for using the AAIC electrostatic spraying machine is in Appendix C.

Saturated Wipe Application.

A robotic wipe machine, Swiping Automated Machine (SAM; Engineerable LLC, Atlanta, GA), was constructed for this project. This wipe machine was programmed to apply a saturated wipe with a constant downward force (1.000 kg mass load at an overall horizontal speed of 0.56 cm/s over an area of 4 cm^2) back and forth over a single, horizontally-positioned stainless steel coupon. A step-by-step protocol entitled "SAM Instructions for Swiping" for using this machine is in Appendix D.

Sterilized scissors were used to cut 24.1 cm x 18.3 cm wipes (100% polyester, Best Sanitizers, Inc., Penn Valley, CA) into four equal rectangles, 6.03 cm x 4.58 cm ("wipes"). Figure 3.12 shows a 4X magnification of a wipe section.

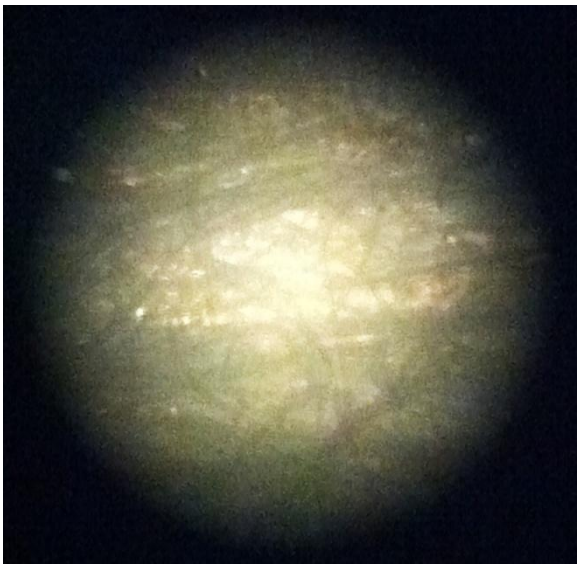

Figure 3.12 A 4X magnification of a wipe used in the sanitizing wipe experiments, showing the fibrous, 100% polyester material. *Photo credit: Morgan Bankston and Taylor Elser.*

Treatment solutions were prepared as mentioned previously in sterile 300 mL glass jars. The saturation volume for each wipe, 2.6 mL of treatment solution, was placed into a sterile 15 mL centrifuge tube (#89039-664, 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 treatment solution at the bottom of the tube 5 min prior to wiping, using sterile forceps (#Z168777, Sigma-Aldrich, Co. LLC, St. Louis, MO), so as to saturate the entire wipe.

Two suction cups, facing upward and fitted on a moveable platform with a vacuum hose connection, held the stainless steel coupons in place during wiping. A robotic arm fixed with a magnetically-removable plastic cube performed the swiping motion. A wipe saturated with a treatment solution 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 x 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/elut combo solution.

Calculation of Log Virus Removal/Inactivation and Statistical Analysis.

The lower limit of detection was 3.30 logs of virus, or 2,000 viral PFUs, for all replicates with hydraulic, AAIC electrostatic, and wipe applications. As mentioned previously, this detection limit was lowered to 1.10 logs of virus with the PEG precipitation procedure, which was performed for some of the treatments of the wipe experiment if no virus was detected on the zero dilution plate.

For each application method, sanitizers and controls were prepared and applied on three different days to each of three test samples and negative controls. Average MNV-1 log reductions (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 post-treatment for each sample in each replicate was subtracted from the average log PFU MNV-1/mL recovered from recovery controls. Data were statistically analyzed using a complete two-way analysis of variance (ANOVA) (SAS 9.3, 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. The variances across the treatments, however, were dissimilar, and a "Variance Components" structure was assumed in order to allow for and estimate different variances for each treatment. Differences between treatment log reduction means were considered significant when the P-value of the difference was less than 0.05.

CHAPTER 4

RESULTS

Optimization of Neutralization and Elution Procedures.

As shown in Table 4.1, the novel neut/elut combo outperformed letheen broth, DE broth, and DE broth with sodium bicarbonate in neutralization capability in a preliminary study with 2-3 samples per sanitizer treatment. Although the novel neut/elut combo did not outperform DMEM with added sodium thiosulfate for the neutralization of 200 ppm chlorine or DMEM for the neutralization of 5% LEV / 2% SDS, the active neutralizing agent for chlorine, sodium thiosulfate, and for 5% LEV / 2% SDS, sodium bicarbonate, were also included in the formulation of the neut/elut combo. The neut/elut combo eluted more dried virus from stainless steel coupons than 0.1 M high salt PBS by 0.20 log PFU MNV-1/mL. Thus, the neut/elut combo was chosen as the neutralization and elution buffer solution of choice for the sanitizer application studies.

Table 4.1 Results from a preliminary study to optimize the neutralization and elution buffer used in the sanitizer application studies.

NEUTRALIZATION TESTS	Number of Samples (n)	Average Virus Recovered (Log PFU MNV-1/mL)	Virus Stock (Log PFU MNV-1/mL)	Log Virus Stock – Average Log Recovered
DE broth, 15 mL				
Alpet D2	2	7.37	8.00	0.63
200 ppm Chlorine	2	7.48	8.00	0.52
DE broth with sodium bicarbonate, 15 mL				
5% LEV / 2% SDS	2	7.53	8.00	0.47
DMEM, 15 mL				
5% LEV / 2% SDS	2	7.85	8.00	0.15
DMEM with sodium thiosulfate, 15 mL				
200 ppm Chlorine	2	8.00	8.00	0.00
DE broth with sodium bicarbonate, 15 mL				
5% LEV / 2% SDS	2	7.53	8.00	0.47
Letheen broth, 10 mL				
Alpet D2	2	7.64	8.00	0.36
Letheen broth, 15 mL				
Alpet D2	2	7.93	8.00	0.07
Neut/elut combo[1], 15 mL				
Alpet D2	3	8.00	8.00	0.00
200 ppm Chlorine	2	7.92	8.00	0.08
5% LEV / 2% SDS	2	7.83	8.00	0.17
ELUTION TESTS	Number of Samples (n)	Average Virus Recovered (Log PFU MNV-1/mL)	Virus Stock (Log PFU MNV-1/mL)	Log Virus Stock – Average Log Recovered
Neut/elut combo[1], 15 mL	3	7.32	8.00	0.68
0.1 M High salt PBS	2	7.12	8.00	0.88

[1]Neut/elut combo solution [letheen broth with tween 80 (Neogen Corporation, Lansing, MI) plus 1 M NaCl, 0.01 g sodium thiosulfate/500 mL, and 2.0 g sodium bicarbonate/500 mL]

Recovery of MNV-1 from Stainless Steel Coupons.

Using the novel neut/elut combo solution to recover MNV-1 from stainless steel coupons, average recoveries and corresponding virus stock titers are listed in Table 4.2 for the hydraulic spray (HYD), AAIC electrostatic spray (ESS), and wipe (WIPE) application methods.

Each replicate includes five recovery control samples, except for WIPE REP 2, where only four samples were used due to a fungal contamination of one sample during incubation.

Table 4.2 Average MNV-1 stock titer and average recovery control titers obtained after drying MNV-1 on stainless steel coupons and elution with the neut/elut combo during each experimental replicate.

Treatment Type and Replicate Number	Avg. Virus Stock (log PFU MNV-1/mL)	Avg. Recovery Control (log PFU MNV-1/mL)	Standard Deviation of Recovery Control Values
HYD REP 1	8.34	6.95	0.189
HYD REP 2	8.63	6.95	0.042
HYD REP 3	8.29	7.72	0.055
ESS REP 1	8.02	7.62	0.126
ESS REP 2	6.06	6.27	0.055
ESS REP 3	6.50	5.93	0.055
WIPE REP 1	8.77	7.83	0.115
WIPE REP 2	7.93	8.13	0.091
WIPE REP 3	8.24	8.42	0.044

MNV-1 Inactivation on Stainless Steel following Sanitizer Treatment with Hydraulic Spray.

Overall, the 5% LEV / 2% SDS sanitizer treatment resulted in a significantly greater reduction in MNV-1 than all other treatments ($p < 0.02$) using hydraulic spray, as shown in Figure 4.3 and Table 4.4. The average (n = 9) log PFU MNV-1/mL reduction due to 5% LEV / 2% SDS was 2.71. Alpet D2 sanitizer treatment resulted in a significantly greater reduction of MNV-1 than all other treatments except 5% LEV / 2% SDS ($p < 0.001$). The average (n = 9) log PFU MNV-1/mL reduction due to Alpet D2 was 2.23. There was a significant day to day variation, however, and on one day of hydraulic application Alpet D2 resulted in a significantly greater reduction of MNV-1 than all other treatments ($p < 0.001$) (data not shown). The average (n = 8) log PFU MNV-1/mL reduction for 200 ppm chlorine, in widespread use in the food industry as a spray sanitizer *(15)*, 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 a surfactant to sterile tap water does not increase its ability to remove MNV-1 from a surface under the conditions of this experiment.

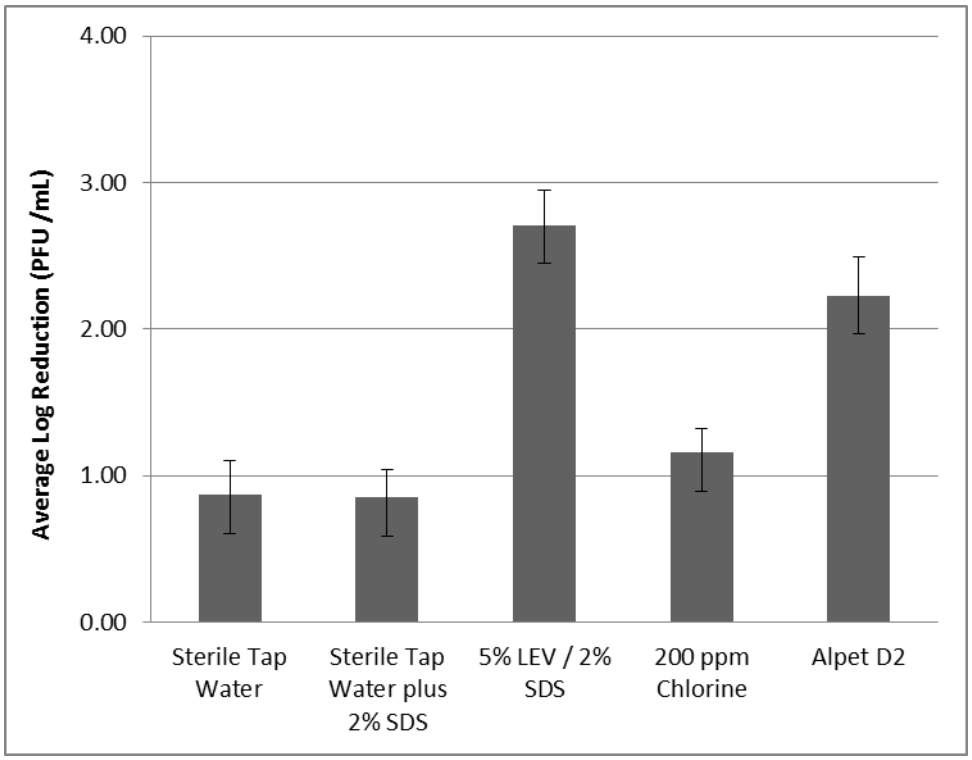

Figure 4.3 Log reduction of infectious MNV-1 on stainless steel following hydraulic spray application of liquid sanitizers or controls. Error bars indicate standard deviations.

Table 4.4 Average log reductions of infectious MNV-1 and standard error for treatments applied using a conventional, hydraulic spray apparatus.

HYDRAULIC SPRAY APPLICATION		
Treatment Liquid	**Mean Log Reduction of MNV-1 due to Treatment**	**Standard Error**
Sterile Tap Water	0.87^{cd}	0.09
Sterile Tap Water plus 2% SDS	0.85^{d}	0.10
5% LEV / 2% SDS	2.71^{a}	0.07
200 ppm Chlorine	1.16^{c}	0.09
Alpet D2	2.23^{b}	0.07

Different letters indicate statistically different values for log reductions.

MNV-1 Inactivation on Stainless Steel following Sanitizer Treatment with Air-Assisted, Induction-Charged Electrostatic Spray.

An overall comparison between sanitizing treatments is shown in Figure 4.5 and Table 4.6. The largest MNV-1 reductions were observed after treatment with 5% LEV / 2% SDS or 200 ppm chlorine, which were both significantly greater than all other treatments ($p < 0.005$).

However, there was no difference in MNV-1 log reductions observed after treatment with 5% LEV / 2% SDS or 200 ppm chlorine ($p = 0.57$). Treatment with Alpet D2 and sterile tap water plus 2% SDS provided a significant difference in MNV-1 reductions ($p = 0.0004$), but neither were significantly different than treatment with sterile tap water ($p > 0.79$). Thus, Alpet D2 has no greater ability to reduce infectious MNV-1 viral load under the conditions of this experiment than does sterile tap water, and reduces viral load to a significantly lesser extent than sterile tap water plus 2% SDS.

Again, day to day variation was apparent with AAIC electrostatic spray application (data not shown). For example, on day three, 200 ppm chlorine was not significantly different from sterile tap water ($p = 0.31$). Also, on day one, 5% LEV / 2% SDS and 200 ppm chlorine did not result in a significantly greater reduction than the other treatments ($p > 0.32$). Day one resulted in the most statistically significant differences between the treatments when comparing with the other days [on day one only the two pairs Alpet D2 and sterile tap water plus 2% SDS ($p = 0.007$), and sterile tap water and 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 can be estimated that the lower titer virus stock used in days two and three of the AAIC electrostatic replicates increased the sanitizer treatment effectiveness for all treatments except sterile tap water plus 2% SDS.

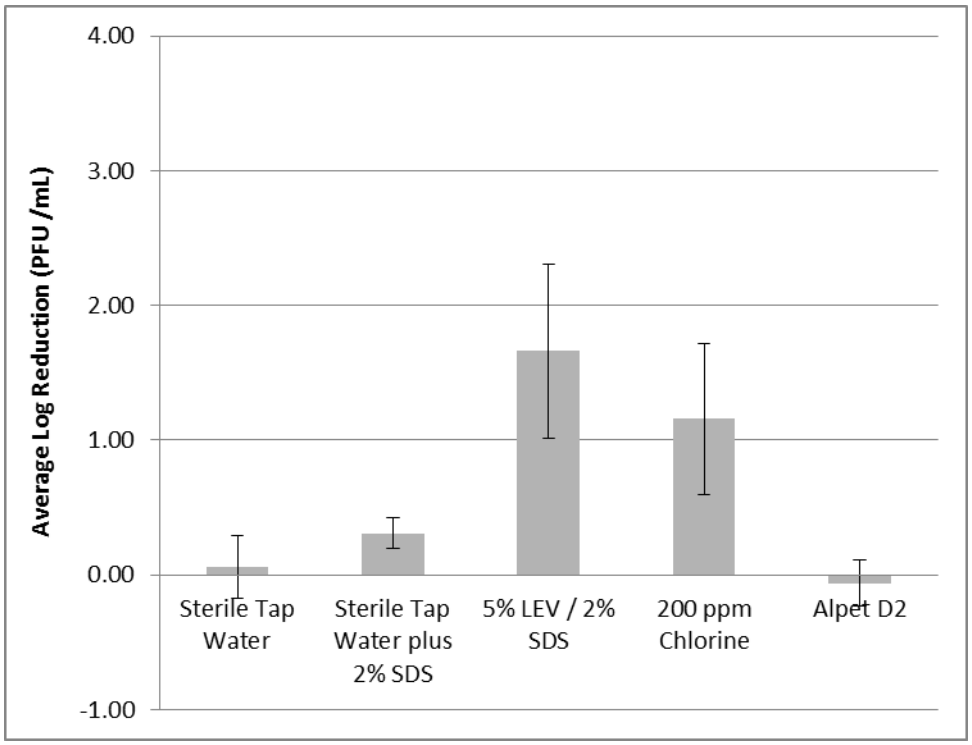

Figure 4.5 Log reduction of infectious MNV-1 on stainless steel following AAIC electrostatic spray application of liquid sanitizers or controls. Error bars indicate standard deviations.

Table 4.6 Average log reductions of infectious MNV-1 due to treatments and standard error for treatments applied with AAIC electrostatic spray technology.

AAIC ELECTROSTATIC SPRAY APPLICATION		
Treatment Liquid	**Mean Log Reduction of MNV-1 due to Treatment**	**Standard Error**
Sterile Tap Water	0.06[bc]	0.09
Sterile Tap Water plus 2% SDS	0.31[b]	0.04
5% LEV / 2% SDS	1.66[a]	0.25
200 ppm Chlorine	1.16[a]	0.22
Alpet D2	-0.06[c]	0.07

Different letters indicate statistically different values for log reductions.

MNV-1 Inactivation on Stainless Steel following Sanitizer Treatment with Saturated Wipes.

The 5% LEV / 2% SDS and 200 ppm chlorine treatments resulted in significantly greater MNV-1 reduction than the three other treatments for the robotic wipe application ($p < 0.0001$), as shown in Figure 4.7 and Table 4.8. 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 PEG precipitation procedures). 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 reduction 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 increase benefits as a sanitizer applied as a wipe over sterile tap water, and likewise adding a surfactant to the sterile tap water revealed no increase in viral reduction for this application.

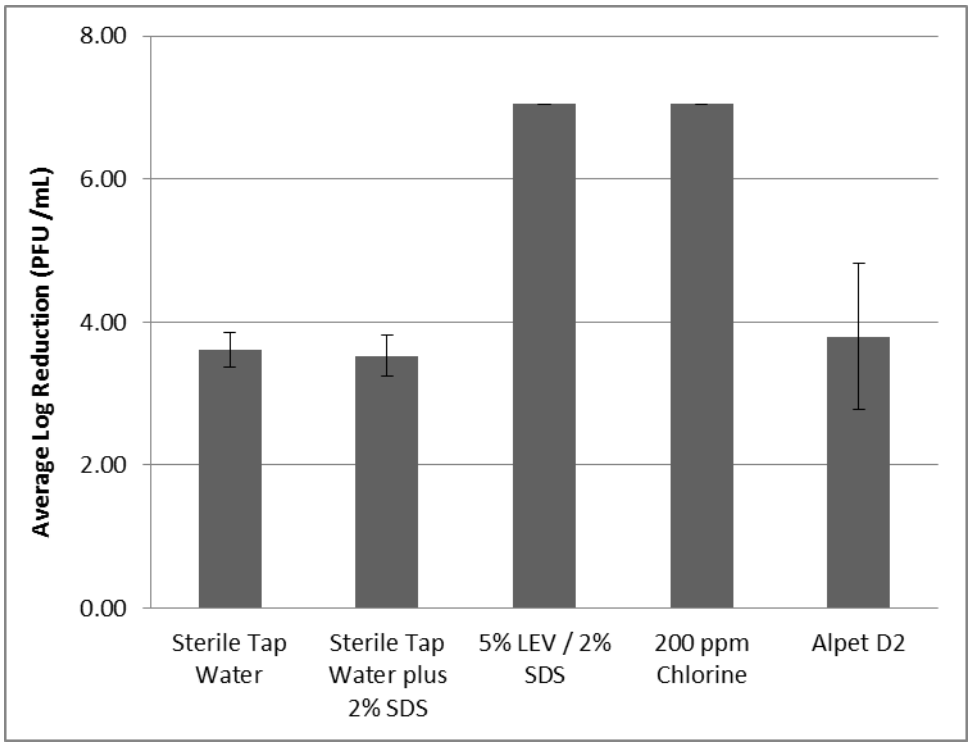

Figure 4.7 Log reduction of infectious MNV-1 on stainless steel following wipe application of liquid sanitizers or controls. Error bars indicate standard deviations.

Table 4.8 Average log reductions of infectious MNV-1 and standard error for treatments applied with a robotic wiping device.

WIPE APPLICATION		
Treatment Liquid	**Mean Log Reduction of MNV-1 due to Treatment**	**Standard Error**
Sterile Tap Water	3.61[b]	0.09
Sterile Tap Water plus 2% SDS	3.53[b]	0.10
5% LEV / 2% SDS	7.05[a]	ND
200 ppm Chlorine	7.05[a]	ND
Alpet D2	3.80[b]	0.49

Different letters indicate statistically different values for log reductions.
ND = not determined

CHAPTER 5

DISCUSSION

MNV-1 Attachment to Stainless Steel.

According to Deboosere et al. (2012), MNV-1 is the surrogate showing the most promise as a model for prediction of the adhesion of human NoV to stainless steel *(28)*. In this study, over 250 stainless steel coupons were observed post-inoculation with high titer MNV-1. Visually, there appeared to be an initial drying period where the virus inoculum formed a thin film on the stainless steel surface but remained wet. The inoculum was considered visibly dry when it ended a second drying phase to form a dry film on the surface (Figure 5.1).

Figure 5.1 A stainless steel coupon with MNV-1 inoculum considered to be visibly dry, having passed through two stages of drying.

Under the experimental conditions in this study (RH = 26-63%; T = 20.5 ±1.3°C), inoculum drying required at least 50 min and up to 185 min. Drying time usually increased with an increase in

relative humidity, but not for all cases. For example, during one replicate the RH of the room was 60% with a total drying time of up to 185 min, while in another replicate, the RH was 63% with a drying time of only 70 min. This phenomena may be explained by other factors, such as air flow within the room which could not be controlled on the biosafety level 2 laboratory bench *(90)*. Future studies should consider using a laminar flow hood to dry the coupons, where the air flow is better controlled, if possible. The variations in relative humidity and air flow with regard to inoculum drying in this experiment suggest that any standardized methods developed for sanitizer effectiveness studies on hard, non-porous surfaces would benefit from considering temperature, air flow, and relative humidity in the drying environment during experimental design.

Since the drying time and relative humidity differences between days do not correlate with the differences in the results for each treatment, a complete normalization of relative humidity and drying time for MNV-1 inoculated onto stainless steel coupons, used in sanitizer studies, is likely unnecessary, but further data is needed for confirmation. Instead, the titer of the virus stock may be a more important factor to control. Looking at the variation of the results after AAIC electrostatic replicates, which differed the most out of all applications, the virus stock titer used on day one was of 8.02 log PFU MNV-1/mL, whereas, on days two and three, virus stock titers ranged between 6.06 and 6.50 log PFU MNV-1/mL. On day one, the mean log reduction for 5% LEV/ 2% SDS, for example, was only 0.48 log, whereas on day two it was 2.63 log and on day three, 1.87 log. Therefore, it is probable that contamination with a lower virus stock titer is more easily sanitized with AAIC electrostatic spraying, and possibly other applications. Further studies are needed to confirm this hypothesis.

The Efficacy and Mechanism of Action of 5% LEV / 2% SDS as a Sanitizer Against MNV-1 when Applied to Stainless Steel Surfaces.

The sanitizer which performed the best in all applications (hydraulic spray, AAIC electrostatic spray, and wiping with an impregnated towelette) was 5% levulinic acid plus 2% sodium dodecyl sulfate. The 2.7 pH of 5% LEV / 2% SDS is below the isoelectric point of the norovirus capsid, which is estimated to be between 5.5 and 6.0 for GI and GII norovirus *(40)*. Therefore, the viral capsid will have a net positive charge at pH = 2.7. The low pH of the organic acid is not enough to inactivate MNV-1 alone, as MNV-1 can survive at gastric pHs of < 3, but the combination of the low pH of the organic acid plus the anionic surfactant (SDS) is highly virucidal. Howett et al. (1999) proposed that SDS causes viral proteins of non-enveloped viruses to denature and unfold, ultimately leading to inhibition of infectivity *(44)*, a mechanism supported by others *(74)*. Yet results from this study and from other studies *(17)* reveal MNV-1 resistance to treatment with SDS alone.

As mentioned in Chapter 2, SDS has both a polar end which is negatively charged, and a nonpolar end which is hydrophobic. As an organic acid plus anionic surfactant solution, one proposed mechanism of action against MNV-1 and HuNoV is that the negatively charged ends of SDS molecules react with the positively charged capsid proteins of the virus, which are in greater numbers at a lower pH *(78)*. 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. The hydrophobic tails of SDS molecules, bound to unfolding viral proteins, inhibit infectivity when refolding cannot occur. Micelle formation and hydrophobic interactions between SDS and proteins have been researched extensively by Otzen *(71)*. In fact, SDS itself has been studied as an antimicrobial for both enveloped and non-enveloped viruses *(74)*.

Advantages and disadvantages of the levulinic acid plus SDS sanitizer are shown in Table 5.2.

Table 5.2 The advantages and disadvantages of 5% LEV / 2% SDS as a surface sanitizer.

ADVANTAGES	DISADVANTAGES
• non-irritating to skin • non-corrosive [2] • effective in the presence of organic matter [2] • effective against MNV-1, FCV and many vegetative bacteria [1] • good wetting properties [2] • non-staining [2] • shelf stable [2] • low odor, nonvolatile characteristics [2] • unaffected by water hardness [2] • foaming may be desirable in certain applications	• foam development can occur, which may require several rinses to remove from surface • effectiveness decreases in the presence of alkaline residuals, thus a pre-rinse step may be necessary post-cleaning of a surface [2] • less effective at pH > 3 [2] • high cost [2] • low effectiveness against yeasts and molds [2] • not yet approved for food contact use by the EPA • may corrode iron [2]

Superscripts indicate sources as follows: [1]*(17)*; [2]*(66)*

The strong wetting property of SDS possibly aided in viral reduction for LEV/SDS, which was shown to be the maximum capable through this study's detection methods. This wetting property may have enhanced the efficacy of the sanitizer in the other application methods as well, enabling it to better moisten the stainless steel surface and thus increase sanitizer contact with the viral particles. More contact between sanitizer and virus increases the chance for inactivation of virus, as is shown in practice where sanitizer solution studies, with greater contact between sanitizer and virus, are generally more effective at viral reduction than are application studies *(36)*.

The Efficacy and Mechanism of Action of Alpet D2 as a Sanitizer Against MNV-1 when Applied to Stainless Steel Surfaces.

Alpet D2 (Best Sanitizers, Inc.) showed a significant log reduction of viral load in the hydraulic spray application over both controls and 200 ppm chlorine, but did not significantly

differ from treatment with sterile tap water when used in AAIC electrostatic spray and wipe applications. The manufacturer instructions suggest a coarse spray *(13)*, which coincides with this sanitizer being more effective in the conventional, hydraulic application (average droplet size = 300 µm) than in the AAIC electrostatic spray application (average droplet size = 30 µm). In fact, the isopropyl alcohol-based (58.6%) sanitizer evaporates readily and requires no rinse step post-sanitation *(13)*. With such a small droplet size as in the AAIC electrostatic spray application, it is possible that a portion of the sanitizer evaporated in the 76 cm of space between the spraying nozzle and the stainless steel coupons. The larger droplets of the hydraulic spray only had 42 cm of space in which to evaporate before coming into contact with the stainless steel coupons, supporting the theory that evaporation contributed to the decrease in sanitizer effectiveness.

Another reason the AAIC electrostatic spray application of Alpet D2 was less effective than the hydraulic spray application may be due to a design flaw in the experimental set-up. Alpet D2 sanitizer spray 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 *(50-52)*. The rate of charge conveyed on its spray cloud reached a maximum of -5.2 µA, although the desired charge was -12 µA as routinely achieved on water-based spray. Addition of ions or lecithin to the Alpet D2 formulation may have increased the charge on the spray cloud. Subsequent studies are needed to rule out the possibility that Alpet D2 would perform better as a sanitizer in the AAIC electrostatic spray application if its formulation was modified to increase conductivity. However, the flammability of Alpet D2 makes it less than ideal for use in AAIC electrostatic spraying for safety reasons.

The strong evaporative property of Alpet D2 also affected its ability as a wipe. In fact, Best Sanitizers, Inc., manufactures wipes impregnated with Alpet D2, but does not claim them to

be effective against noroviruses, although the liquid sanitizer holds that claim. Casual tactile observations during this study showed that although the towelettes became saturated with Alpet D2, they became dry faster than the other sanitizer treated towelettes. A wetter wipe creates more mobility of the virus and thus has increased power to physically dislodge dried viral particles from the stainless steel surface, creating a greater contact between the sanitizer and the virus.

As a QAC, the inactivation mechanism of Alpet D2 against MNV-1 is likely the formation of micelle, whereby intact virus is surrounded by sanitizer and aggregated, therefore lowering infectivity by decreasing the number of infective particles. However, QAC are more effective against enveloped viruses than non-enveloped viruses *(83)*. In fact, QAC have often been proven to be ineffective against non-enveloped viruses, including noroviruses *(37)*. One study showed effectiveness of a QAC against feline calicivirus, with greater than a 6 log MPN/mL reduction after a 10 min contact time *(62)*. Isopropyl alcohol has been found to have some virucidal activity when studied with MNV-1 inoculated onto stainless steel after a 5 min contact time *(65)*, so it likely enhances any effect the QAC component has against noroviruses. Certainly, the alcohol contributes to the sanitizer's desirable evaporative effect in low-moisture settings where food industry equipment may be harmed and/or corroded by water, as in the processing of almonds *(29)*.

The advantages and disadvantages of Alpet D2 as a sanitizer are listed in Table 5.3.

Table 5.3 The advantages and disadvantages of Alpet D2 as a surface sanitizer.

ADVANTAGES	DISADVANTAGES
• EPA-approved as a surface disinfectant for food contact surfaces against some viruses and bacteria; FDA-approved for use on food contact surfaces [1] • non-corrosive [2] • long shelf life [2] (2 years) [1] • non-irritating to skin [2] • evaporates quickly and can be used in low-moisture or water-sensitive settings [1]	• high cost • re-wetting of a surface may be necessary due to high evaporation

Superscripts indicate sources as follows: [1]*(13)*; [2]*(66)*

The Efficacy and Mechanism of Action of 200 ppm Chlorine as a Sanitizer Against MNV-1 when Applied to Stainless Steel Surfaces.

The potential of 200 ppm chlorine to inactivate MNV-1 to a greater extent than water alone is greatest when used as a wipe, under the conditions of this study. Although chlorine solutions are the most commonly used sanitizer in many food industries *(15)*, the mechanism for pathogen inactivation is largely unknown. Due to chlorine's strong electron affinity and electronegativity, the mechanism likely involves oxidative damage. For vegetative cells, it is thought that chlorine impairs membranes, disrupting the transport of vital cell nutrients. Other theories suggest an enzyme destruction mechanism, inhibition of glucose oxidation, oxidative decarboxylation of amino acids, or the production of toxic derivatives of cytosine *(66)*. However, for non-enveloped viruses, a study by Shirai et al. (2000) using electron microscopy revealed chlorine degradation of viral particles, resulting in morphological changes in the capsid shell and a leakage of inner viral genomic material *(83)*. Shirai's visual research supports an oxidative action theory where viral capsid proteins are damaged beyond repair.

Since it is a strong oxidizing agent, free, available chlorine is rapidly consumed in the presence of organic matter, in light, and through exposure to air *(15)*. These chemical properties of chlorine, and their negative consequences for HOCl, explain why the sanitizer was

more effective, when compared to the other sanitizers studied, with the wipe application versus the spray applications. A saturated towelette naturally has less liquid surface area exposed to the surrounding environment than does the same amount of solution dispersed into spherical droplets. Therefore, the probability that chlorine will lose sanitizing power when used as a wipe is lower than when chlorine is used as a spray, where the droplets are exposed to light, oxidation, and organic components in the air.

Due to these factors, the levels of free chlorine in a sanitizing solution must be closely monitored. To test for the free, available chlorine in a sanitizing solution, test strips may be used, although a more accurate method is the iodometric, or starch iodide, method. In practice, this method requires time and patience, a disadvantage of using chlorine versus other sanitizers which are more easily prepared. At a pH of between 6 and 6.5, most of the chlorine in solution is HOCl, the most active form, although it is possible to obtain the desired concentrations for food-contact sanitation at room temperature as long as the solution pH is below 10. Each time a new batch of 200 ppm chlorine is to be made, the stock solution of household bleach may have a different level of chlorine depending on storage conditions and how long it has been opened, and tap water pH also varies from day to day. So, it is not possible to simply measure a tablespoon of household bleach into a gallon of water and obtain a perfect 200 ppm concentration, as directions on bleach containers and some sanitation instruction manuals state *(67)*.

Although a solution of 50-200 ppm chlorine is the inexpensive standard no-rinse food-contact sanitizer in the food industry, there are numerous disadvantages to its use, as listed in Table 5.4.

Table 5.4 The advantages and disadvantages of 200 ppm chlorine as a surface sanitizer.

ADVANTAGES	DISADVANTAGES
• effective against a wide range of bacteria, viruses, and fungi [2] • fast-acting [2] • inexpensive [2] • approved for food-contact use by the EPA and the FDA • can be used with hard water [2] • highly researched	• to be effective, the temperature, pH, and free chlorine in the solution needs to be monitored and may take extra time and effort [1,2] • short shelf life of working dilution (dissipates readily) [2] and a 30-day shelf life, once opened, for stock bleach if using household liquid chlorine bleach • corrodes metals [2] • less effective in the presence of organic matter [2] • irritating to skin and mucus membranes [2] • stains surfaces and clothing (important if using as a spray – protective equipment needed) • strong, undesirable odor [1] • sanitation by-products [1]

Superscripts indicate sources as follows: [1]*(15)*; [2]*(66)*

Conventional, Hydraulic Spraying as a Sanitizer Application Method.

Sanitizer sprays are in widespread use in both homes and in the food and foodservice industries. The droplet size (known as the "coarseness"), spray pattern, and pressure can all vary depending on the chosen spray apparatus. With a pressure of 43 psig as used in this study, there is a distinct benefit from the strong mechanical energy able to impact the target surface, dislodging viral particles in preparation for contact with the sanitizer. This energy can also help conventional sprays penetrate into crevices and corners, where pathogens may be harder to reach and/or may collect *(57)*. However, if the pressure in the spray is too strong, viral particles from contaminated areas may become aerosolized and spread around the environment. Another advantage of a hydraulic spray application is that it is often easy to mix in water with the desired sanitizer to obtain an appropriate use dilution, either in the spray reservoir or

through the use of hosing *(57)*. Hydraulic spraying can be fitted for small areas with small amounts of sanitizer, as in fine misting, or for large areas with large amounts of sanitizer, as in pressure washing.

Air-Assisted, Induction-Charged Electrostatic Spraying as a Sanitizer Application Method.

AAIC electrostatic spraying can be compared to mist spraying in terms of pressure, where the target surface becomes wetted by the sanitizer, but the physical force of contact is minimal *(57)*. Because there is a small amount of kinetic energy when applied, this application method works best on pre-cleaned surfaces, where there is no soil to dislodge *(57)*. If the target surface is vertical, as was the case in this study, the bottom of the surface collects the most sanitizer as gravitational forces gradually overcome the electrostatic interactions. Thus, vertical targets may require re-applications of sanitizer depending on the desired contact time, more so than with hydraulic sprays, where the mechanical energy may dislodge the pathogenic particles from the surface, which then collect in the sanitizer pool at the bottom and may be inactivated. A smaller amount of sanitizer, applied with AAIC electrostatic spray, is required to provide the same level of efficacy when compared to hydraulic spray techniques *(63)*, lowering sanitizer costs and environmental impact overall.

The small droplet size both increases the potential for maximum target surface contact and maximum dissipation of potentially hazardous fumes *(57)*, although these fumes are more concentrated to a specific area with AAIC electrostatic spraying than with fine misting alone. Such a small droplet size, when combined with a charge, has the great advantage over conventional spraying for accessing backsides and crevices of otherwise unreachable targets, as shown by Law *(52)*. In fact, sanitizer applied with AAIC electrostatic spray has a 29-times greater deposition rate onto backsides of target surfaces than does hydraulic spray, under the same spraying conditions as used in this study *(63)*. Finally, with such low pressure in application,

AAIC electrostatic spray does not carry a significant risk for dispersing virus from contaminated surfaces into the environment. In fact, AAIC electrostatic spray was studied by Law et al. (2003) as a means to decontaminate the human body in the event of bioterrorism *(54)*, a situation where not dispersing the contaminant to the environment is of utmost importance.

Wiping with Saturated Towelettes as a Sanitizer Application Method.

This study indicates that wiping with impregnated towelettes is a promising means of viral load reduction depending on the chosen sanitizer. Sanitizers with low evaporative properties and high wetting ability appear to work well as a wipe, as was shown through the increased log MNV-1 reduction with 5% LEV / 2% SDS and 200 ppm chlorine over Alpet D2. The wipe material is also important, with thicker wipes of larger pore sizes shown to be more beneficial than thinner wipes with lower pore sizes *(56)*. The disposability of a wipe is both an advantage and a disadvantage. Disposable wipes can decrease the risk for surface-to-surface contamination *(10)* and are both easy and ready for use, but they have a high cost and are less environmentally friendly when compared with non-disposable towelettes. Even if a sanitizer is not available, wiping with water alone can result in a reduction of viral load (mean log PFU MNV-1/mL reduction in this study was 3.61), possibly aided by frictional energy.

Neutralization and Elution Properties of the Novel Neut/Elut Combo.

One solution alone was used to neutralize three different types of sanitizers: an organic acid plus anionic surfactant, chlorine, and an alcohol-based QAC sanitizer, and to elute MNV-1 dried onto stainless steel coupons. The "neut/elut combo" used sodium bicarbonate ($NaHCO_3$) to neutralize the acidity in the 5 % LEV / 2% SDS solution. In solution, sodium bicarbonate forms a bicarbonate ion (HCO_3^-), which is weakly basic and which reacts with levulinic acid. Sodium thiosulfate ($Na_2S_2O_3$) disabled the effectiveness of the HOCl in the 200 ppm chlorine through an oxidation/reduction reaction where thiosulfate ($S_2O_3^{-2}$) reduces hypochlorous acid (HOCl) to Cl^-.

Lecithin in the neut/elut combo neutralized the quaternary ammonium compounds in Alpet D2 (octyl decyl dimethyl ammonium chloride, dodecyl dimethyl ammonium chloride, and dioctyl dimethyl ammonium chloride), while the combination of lecithin and tween 80 neutralized the alcohol component.

One M NaCl in the neut/elut combo provided mechanical action through its crystalline structure to extricate and elute the dried virus from the stainless steel coupons, while tween 80, an emulsifier, likely helped to release the viral particles into the suspension.

Tween 80 and the 1 M NaCl in the neut/elut combo may have also played another role in this study. Throughout the research, some of the neutralization controls were revealing what appeared to be a slightly greater amount of MNV-1 PFU than that in the virus stock titer. This discrepancy may be explained through tween 80 and 1 M NaCl, which likely increase the dispersal of the virus-like particles throughout the neut/elut combo used in the neutralization controls, possibly breaking apart some viral aggregates. If one larger viral aggregate, which would represent one PFU in an assay, was broken into two smaller aggregates, it would then represent two PFUs in an assay. In an aggregation study by da Silva et al. (2010), one strain of GI norovirus did not aggregate in high salt concentration (1 M) *(26)*. If more viral particles are present in the neutralization controls than in the virus stock (where no neut/elut combo is used), then the number of PFUs would also be higher for those controls. The neut/elut combo as a viral particle dispersant may also explain why some recovery controls had higher PFU counts than the virus stock titer, as in day two of AAIC electrostatic spraying and days two and three of wipe application.

CHAPTER 6

CONCLUSIONS

This study revealed the viral inactivation efficacy of three sanitizers, 5% LEV / 2% SDS, 200 ppm chlorine, and Alpet D2, against a human norovirus surrogate, MNV-1, on a stainless steel surface when used with three application methods including hydraulic spraying; air-assisted, induction-charged electrostatic spraying; and wiping with an impregnated towelette. The 5% LEV / 2% SDS sanitizer was most effective in all applications when compared to the other sanitizers and to the controls, although 200 ppm chlorine performed equally as well as 5% LEV / 2% SDS when applied as a wipe. Currently, EPA regulations test chemical solutions against FCV for registration as a food-contact surface disinfectant, but this research chose a more resistant surrogate, MNV-1, and tested for post-treatment infectivity by plaque assay. When reviewing data from viral sanitizer studies, the surrogate virus used, the virus stock titer, the method for quantifying remaining viral load, and possibly the environmental conditions for inoculation should all be considered.

There are several sanitizers on the market to choose from, and as this study points out, many factors need to 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. Furthermore, although one sanitizer and application method pair may be ideal for bacterial contamination, another may be required for norovirus contamination. To maximize sanitizer efficacy against noroviruses, mechanical action plays an important role, as does having a clean surface prior to sanitation. Since some sanitizers, such as Alpet D2, require different contact times for bacteria and viruses and a coarse spray, it is

extremely important to follow manufacturer instructions during application. Misuse by industry of a sanitizer or an application method is a waste of time, energy, and money, and poses a giant public health concern.

By testing the effectiveness of sanitizers against each other and against controls in a manner similar to that of real-world use in the food industry, using the most resistant surrogate, and testing for remaining infective virus rather than mere presence of virus, this research furthered the knowledge base needed to ease the economic and quality of life burdens associated with the large number of norovirus-related foodborne illnesses present today.

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APPENDIX A

TIME SHEET FOR MNV-1 INOCULATION AND COUPON TREATMENT

A detailed form to record the timing of each replicate was used to ensure consistency.

Sample ID	Time Wipe is Saturated	Inoculation Time	Time When Dry	Time Swiped (2X @ 1000g)	Contact Time + Air-Drying	Time put in Neut/Elut
WN	:	:	:	:	0:06	:
W4	:	:	:	:	0:06	:
W5	:	:	:	:	0:06	:
W6	:	:	:	:	0:06	:
WR	n/a	:	:	n/a	n/a	:
SN	:	:	:	:	0:06	:
S4	:	:	:	:	0:06	:
S5	:	:	:	:	0:06	:
S6	:	:	:	:	0:06	:
SR	n/a	:	:	n/a	n/a	:
LN	:	:	:	:	0:06	:
L4	:	:	:	:	0:06	:
L5	:	:	:	:	0:06	:
L6	:	:	:	:	0:06	:
LR	n/a	:	:	n/a	n/a	:
BN	:	:	:	:	0:06	:
B4	:	:	:	:	0:06	:
B5	:	:	:	:	0:06	:
B6	:	:	:	:	0:06	:
BR	n/a	:	:	n/a	n/a	:
AN	:	:	:	:	0:06	:
A4	:	:	:	:	0:06	:
A5	:	:	:	:	0:06	:
A6	:	:	:	:	0:06	:
AR	n/a	:	:	n/a	n/a	:

APPENDIX B

INSTRUCTIONS FOR HYDRAULIC SPRAY APPARATUS

Step-by-Step Instructions for Spray Apparatus Use – Hydraulic Nozzle

1. Clear out rusty water into white bucket.
    a. Fill liquid-holding reservoir with sterile tap water (1/2 gallon).
    b. Open blue valve and orange valve so that water flows.
    c. Move steel robotic arm to center position and check that the yellow tubing is connected. You will not use a nozzle yet.
    d. Turn pump on and let run until clear and then a little extra.
    e. DO NOT LET PUMP RUN DRY FOR MORE THAN 30 SECONDS.
    f. DO NOT LET PRESSURE GO ABOVE 43 PSI.
2. Move arm back to resting position by flicking black switch on top and reset digital timer to zero.
3. Carefully attach hydraulic nozzle (check strainer for cleanliness periodically). Slotted orifice of nozzle tip needs to be parallel to steel arm.
4. Make sure that desired distance between nozzle and target is correct. (16 ½ inches or 42 cm from middle of target to tip of nozzle)
5. Fill liquid-holding reservoir with desired liquid.
6. Let liquid run for a little bit through nozzle to clear out all water.
7. Adjust pressure as needed (43 psi). Slowly turn brass knob!
8. Turn exhaust fan motor and main blower high speed on.
9. Check timing of arm. (must be between 5800-6200 milliseconds)

10. Spray gloves with 70% alcohol.
11. Load coupons (3 samples + 1 negative control) onto target-holding arc.
12. Turn pump on. If no liquid flows through system, it may be necessary to momentarily use compressed air in the reservoir container to initially prime the pump.
13. Allow a few seconds to allow air to spit out of tubing and ensure liquid flow.
14. Flick switch to start steel arm and start timer.
15. At end of dual pass, turn pump off to conserve liquid.
16. Allow coupons a 5 minute contact and a 1 minute air dry time.
17. At end of replicate, move arm to center and let liquid run out.
18. Rinse with sterile tap water (1/2 gallon).
19. Blow air out until dry.
20. Collect any drips with a Kim wipe.

If finished with the spray apparatus, spray down the chamber with appropriate, noncorrosive sanitizer, keeping fan on. Let sit a few minutes, then turn fan off.

APPENDIX C

INSTRUCTIONS FOR AAIC ELECTROSTATIC SPRAY APPARATUS

Step-by-Step Instructions for Spray Apparatus Use – Electrostatic Nozzle (ESS)

1. Attach the pink tube to the top of the robotic arm using wrench.
2. Clean electrostatic nozzle.
    - Using a chem wipe, wipe inside/outside of nozzle and ensure the tiny black electric-contactor tube freely moves up/down.
3. Carefully attach ESS nozzle: Clear liquid tube goes on top. Green grounding wire clips onto clear tubing where the brass is. Red wire clips into red wire on arm.
4. Ensure desired distance between nozzle and target is correct. (30 inches or 72 cm from middle of target to tip of nozzle)
5. Fill glass jar with a stir bar and desired liquid (start with sterile tap water) using a funnel. Do not get electrical equipment wet. Keep valve closed.
6. Turn power strip and stir bar on.
7. Check timing of arm. (needs to be 5800-6200 milliseconds; turning knob to the left increases time)
8. Turn exhaust fan motor and main blower high speed on.
9. Slowly adjust atomizing-air pressure to 30 psi using black knob, as read on pressure gauge at end of robotic arm, and let air flow through the apparatus for 5 minutes.
10. Open blue valve slowly.
11. Check liquid flow. (100ml/min with no air bubbles stuck in display; if tiny ball is stuck at bottom, stop liquid flow and give it a rest, then re-start – may take up to a 15-min break)

12. **Turn charge on using tan Lambda box. Push 8 . 0 6 enter (or appropriate value to achieve a spray cloud current of -12 micro amps). Make sure to push the decimal place!!! Venus box should also be on.**
13. **Turn first orange digital multimeter on to 1000 volts.**
14. **Turn second orange digital multimeter on to 200 micro amps.**
15. **Using needle on the end of second orange converter's red wire and apparatus for this purpose, check that the charge cloud is coming out between -12 and -15 micro amps.**
16. Close blue liquid valve and refill glass jar if needed.
17. Spray gloves with 70% alcohol.
18. Load 4 coupons (3 for treatment and 1 for a negative control) using sterile alligator clips.
19. Slowly open blue liquid valve, and check liquid flow and pressure.
20. Wait a few seconds to allow air to spit out of tubing.
21. Flick switch to start steel robotic arm, at the same time starting the 6-minute timer. Flick switch a total of six times back-to-back for a total of 36 seconds of spray time.
22. At end of six dual-pass swings, turn liquid valve off to conserve liquid. Pour new liquid in for the uncharged replicate.
23. Let coupons stay in chamber for the remaining 5 min. contact time plus 1 min. of air drying time.
24. Place each sample, negative control plus a recovery control into a neut/elut combo tube. Rock 3 times.
25. Set spray charge to zero. (0 enter)
26. Repeat steps 17-24 for uncharged samples.
27. Dump liquid out of glass bottle into a dump beaker (keep valve open).

28. Close valve and fill glass jar with sterile tap water (between LEV/SDS and bleach; bleach and AD2), rinsing three times into dump beaker, or solution (2% SDS and LEV/SDS).
29. Let water (Bleach, AD2) or solution (2% SDS, LEV/SDS) flow for a few minutes through system.
30. Close blue liquid valve. Tilt glass jar to get out remaining water (Bleach, AD2).
31. Pour in new treatment liquid and allow to run through the system, uncharged, then reset charge and confirm (repeat steps 9-18).

AT END OF TREATMENTS:

32. Let air blow through for a few minutes.
33. Spray out main chamber with water and Alpet D2.
34. Let sit for a few minutes then turn exhaust and blower off.
35. Turn black air pressure knob off.
36. Turn top power strip off.
37. Disconnect nozzle and place face down in beaker home.

(Steps 12-15 only needed if charge is ON. To turn charge off, type in 0 and enter into main charge box.)

APPENDIX D

INSTRUCTIONS FOR ROBOTIC WIPING MACHINE, SAM

SAM Instructions for Swiping

FIRST STEP: SETTING UP THE MACHINE USING A "TRIAL" SURFACE AND A "TRIAL" WIPE

1. Power should be turned off. There is an on/off switch in the rear of the control box on the left-hand side.
2. Place surface to be swiped onto the smaller suction cup platform (the larger suction cup platform is for touching, not swiping). Center the sample surface on the platform, then turn on the vacuum, which should be connected to the platform using rubber tubing.
3. Drape the material to be swiped over the plastic piece which attaches magnetically to the robot. Use a black rubber ring to attach the material to the plastic piece, then magnetically attach the covered plastic piece to the moving piece on the robot.
4. If necessary, move the base so that the platform and sample are centered underneath the swiper.
5. Using the hand wheel at the top of the robot (white), lower the moving end until it is ~3 mm from the surface of the surface to be swiped. NOTE: Make sure that the two surfaces are NOT touching!
6. Loosen the switch on the vertical column (red piece), and move it down until the center black line is aligned with the top edge of the carriage on the vertical axis. Tighten the switch in that position.
7. Loosen the switch on the horizontal column so that you have room to adjust the horizontal motion of the swiping. Use the silver knob on the robot to adjust the start

and end points for swiping, then tighten the two positions on the robot according to the small metal sensors.

8. Make sure that the swipe on/off switch is in the ON position.
9. Turn the power on using the switch behind the control box.
10. Wait until the force meter at the front of the control box initializes and shows a force reading. If the force is not close to zero, check that the sample surface and the swiping surfaces are not touching. If the surfaces are not touching, use the TARE button to re-zero the force meter. NOTE: Both setpoint lights should be off. (These lights are on the left side of the force meter, between the buttons and the display.)
11. Press the green start button on top of the control box. The vertical slide will move up to the switch, and back down about one centimeter to the start position. Carefully examine the surface to be swiped and the robot from several angles to ensure a proper fit.
12. Setpoint 1 is the first option that appears. It must always be a value lower than the desired contact force. Setpoint 1 tells the motor to slow down before it reaches the desired force, which will be Setpoint 2. If the Setpoint 1 is much smaller than the desired force, the force will be more accurate but SAM will take longer to achieve that force. If Setpoint 1 is closer to the desired force, SAM will reach that force quicker. If Setpoint 1 is too close to the desired force, SAM may overshoot. Choose your Setpoint 1 value _____ g and your Setpoint 2 value (desired force) _____ g.
13. Press SETPTS on the force readout meter. Setpoint 1 will appear first.
14. Set Setpoints 1 and 2: Press >/TARE button to move the selection to the right as needed. Press ^/NT/GRS to change the value of the selected digit. Once Setpoint 1 is correct, press SETPTS again to move to Setpoint 2.

15. Press SETPTS several more times until RUN is displayed on the screen.
16. If needed, press TARE again.
17. Press the green start button to test SAM.

SECOND STEP: RUNNING YOUR SAMPLES.

1. Load your sample surface and turn on the vacuum.
2. Load your swiping surface onto the white plastic piece and attach with a black plastic ring.
3. Attach the swiping surface to SAM using the magnets.
4. Press the TARE button.
5. Press the green start button. NOTE: To mimic a back-and-forth swiping motion, after one swipe is completed, turn the vacuum off and rotate the sample surface 180 degrees, turn the vacuum back on, and press the green start button again.
6. Turn vacuum off and remove your sample surface.

WHEN FINISHED: Detach swiping surface. Turn SAM off using the power switch in the rear of the control box.

APPENDIX E

PLAQUE ASSAY PROTOCOL

MNV Plaque Assay Protocol

Pre-Day One: Make agarose for day one and day two, and autoclave on liquid cycle (#1). Remove from autoclave promptly, tighten caps and place on counter at room temp. Portion out the 1.0% agarose into separate glass bottles, one bottle for one-two trays (36-72 plates). Portion out the 0.75% agarose into one glass bottle for every two trays (72 plates). See chart below. For 150 mL of 1.0% agarose, add 1.50 g, and for 250 mL of 0.75% agarose, add 1.88 g, using DI water.

Day One:	per plate	per tray	1 tray	2 trays	3 trays	4 trays	8 trays
1.0% agarose	1.5 mL	54 mL	75 mL	150 mL	75 mL 150 mL	150 mL 150 mL	150 mL 150 mL 150 mL 150 mL
2XMEM	1.5 mL	54 mL	75 mL	150 mL	225 mL	300 mL	600 mL
1XMEM	0.4 mL	14.4 mL	25 mL	50 mL	75 mL	100 mL	200 mL
Day Two:							
0.75% agarose	3 mL	108 mL	150 mL	250 mL	150 mL 250 mL	250 mL 250 mL	250 mL 250 mL 250 mL 250 mL
1.1% neutral red			1.65 mL	2.75 mL			2.75 mL per bottle

Sterilize scissors with 70% ethanol and 15 min of UV light to cut off tips of pipette tips for aspiration. Empty aspiration waste jar and bleach contents until liquid turns pale yellow, then autoclave contents and dump down drain. Make 1x MEM dilution tubes with 900 µL in each tube.

Day One

1. Look at cells under the microscope to ensure 80-90% coverage (check one plate from each tray).

2. Remove samples to be assayed from -80°C freezer and place on counter to thaw until they are at room temperature. Remove dilution tubes from fridge and place in the hood. Portion out and label dilution tubes, checking each one to ensure that it contains 900 µL of 1x MEM.

3. If necessary, heat 1% SeaKem agarose in the microwave for 3-5 min, ensuring that the top is loosened enough to let steam release. Watch the bottle so that it does not boil out. Let cool for five minutes before handling. While agarose is cooling, ethanol the incubator trays and wipe with paper towels. Also check to see if there are enough pipette tips in the hood and that the trash is emptied so that you have room to place your trash.

4. At least 45 min prior to infection, place infection media (1x MEM) and 2x MNV MEM in the 37°C water bath, and place 1% SeaKem agarose in the 45°C water bath. Make sure that the water in the bath covers up to where the media is in the bottles. If needed, 1x MEM can be made from 2x MEM and sterile Milli-Q water. Portion out the 1x MEM into 50 mL tubes, and the 2x MEM into glass jars containing portions which match your agarose amounts.

5. Make dilutions and label plates (one tray at a time, resting the trays on a blue experiment mat). Save 6 plates for plaque assay controls: 2 each of NO AG, NO VS; NO AG, VS-2; AG, NO VS.

6. Aspirate growth medium from the plates, one tray at a time. Gently add 400 µL of 1x MEM to the center of each plate using a micro-pipettor. Rock the tray then put the 1x MEM back in the water bath. The NO AG plates should get around 3 mL of 1x MEM.

7. Plate 100 µL of sample dilutions, in duplicate. Rock tray. Place tray in the top, viral 37°C incubator with 5% CO_2 for 1 h, rocking the tray every 15 min. Record the start time, end time, and 15 min intervals where you can view them, preferably on the timing sheet.

8. After 1 h, aspirate off infection medium (except on the NO AG controls). Quickly mix equal amounts of 1% agarose and 2x MNV MEM, and add 3 mL to each dish using a 25 mL pipette. Check for spills and clean using an alcohol-sprayed paper towel.

9. Leave trays in incubator for 48 ± 5 h.

Day Two

10. Under the microscope, examine the cells on the "no agarose, virus stock -2," "no agarose, no virus" and "agarose, no virus" controls to ensure that the virus has had ample time to infect the cells. To proceed, heat 0.75% SeaKem agarose in the microwave for 3-5 min (if needed), ensuring that the top is loosened enough to let steam release. Watch the bottle so that it does not boil out. Let cool for 5 min before handling.

11. At least 45 min prior to adding second layer, place neutral red in the 37°C water bath and 0.75% agarose in the 45°C water bath. Ensure that the neutral red has not expired and has been kept in the fridge with foil covering it – it goes bad easily.

12. Add 1.1% neutral red (see chart above) to the 0.75% agarose and mix well with pipette and by swirling the jar. Using a 25 mL pipette, add 3 mL of this mixture to each dish. Let sit in the hood for 5-10 min to set, then place back in the same incubator. Check for any spills and wipe up with an alcohol-sprayed paper towel.

13. After 4-8 h, count plaques on plates that show 5-50 plaque-forming units. Record counts in appropriate Excel file.

APPENDIX F

AN OVERVIEW OF CONTROLS USED IN THIS STUDY

Control Overview

Sterile Tap Water Treatment: To show how much virus is simply washed off versus sanitized. Also, to show how much virus can be removed with water alone.

Sterile Tap Water plus 2% SDS Treatment: To show how much virus is washed off with water plus surfactant. Also, to show how much virus can be removed with water plus an inert (against viruses) surfactant alone, especially as compared to the LEV/SDS treatment.

Plate Recovery Control (untreated, inoculated coupons neutralized and eluted in the same manner as treated coupons): To quantify how much dried virus can be recovered from the stainless steel coupons after immersion in neutralizer/elution buffer solution with 30 s of vortexing. The average of the five recovery controls will be used as a baseline of comparison to treated coupons.

Neutralization Control (neutralized sanitizer with added virus stock): To ensure that the neutralizer in fact stops the sanitizer action, so that the contact time for each sanitizer treatment is consistent and equivalent.

Cytotoxicity Control (solution from treated, uninoculated coupons that are neutralized and eluted, both with and without virus stock): To ensure that the end solution containing virus is not toxic to the host cells, nor does it inhibit or hinder viral infection.

Cell Viability Control (infection media plated on cells): To show that the infection media, which is used to make dilutions, is not harming the cells/is sterile and also to show that the cells remain viable throughout the plaque assay. The Cell Viability Control is also a form of a Plaque Assay Control, with agarose.

Plaque Assay Controls (cell plates with and without virus, but no agarose): To make sure that the virus has enough time to infect the cells before counting plaques.

Virus Stock Titer (virus stock in solution, not dried on coupons): To show the original titer of the virus stock used in each experiment and to compare with neutralization and cytotoxicity controls.

APPENDIX G

CALCULATION OF THE AVERAGE DROPLET CHARGE-TO-MASS RATIO CARRIED ON THE SPRAY CLOUD FOR THE AAIC ELECTROSTATIC SPRAY APPLICATION

for Sterile Tap Water, Sterile Tap Water plus SDS, LEV/SDS, and 200 ppm Chlorine:

Step 1. Converting the flow rate from mL/min to g/s.

liquid flow rate = 100 mL / 1 min
assuming density of spray liquid is approximately that of water, 1 g / 1 mL
1 min = 60 s

$$\frac{100\ \text{mL}}{1\ \text{min}} \left| \frac{1\ \text{min}}{60\ \text{s}} \right| \frac{1\ \text{g}}{1\ \text{mL}} = 100\ \text{g / 60 s flow rate}$$

Step 2. Converting the spray cloud charge flow rate from µA to mC/s.

measured charge on spray cloud = - 12 µA
1 A = 1 C/s

$$\frac{-12\ \mu\text{A}}{} \left| \frac{1\ \text{A}}{10^6\ \mu\text{A}} \right| \frac{1\ \text{C}}{1\ \text{As}} \left| \frac{10^3\ \text{mC}}{1\ \text{C}} \right. = -0.012\ \text{mC/s}$$

Step 3. Combining liquid mass flow rate and spray cloud charge flow rate to get charge-to-mass ratio in mC/kg.

$$\frac{-0.012\ \text{mC}}{1\ \text{s}} \left| \frac{60\ \text{s}}{100\ \text{g}} \right| \frac{1000\ \text{g}}{1\ \text{kg}} = \boxed{-7.2\ \text{mC/kg}}$$

for Alpet D2 (non-conductive, alcohol-based liquid):

Step 1. Converting the flow rate from mL/min to g/s.

liquid flow rate = 100 mL / 1 min
density of spray liquid = 0.8 g / 1 mL
1 min = 60 s

$$\frac{100\ \text{mL}}{1\ \text{min}} \left| \frac{1\ \text{min}}{60\ \text{s}} \right| \frac{0.8\ \text{g}}{1\ \text{mL}} = 80\ \text{g / 60 s flow rate}$$

Step 2. Converting the spray cloud charge flow rate from µA to mC/s.

measured charge on spray cloud = - 5 µA
1 A = 1 C/s

$$\frac{-5\ \mu\text{A}}{} \left| \frac{1\ \text{A}}{10^6\ \mu\text{A}} \right| \frac{1\ \text{C}}{1\ \text{As}} \left| \frac{10^3\ \text{mC}}{1\ \text{C}} \right. = -0.005\ \text{mC/s}$$

Step 3. Combining liquid mass flow rate and spray cloud charge to get charge-to-mass ratio in mC/kg.

$$\frac{-0.005\ \text{mC}}{1\ \cancel{\text{s}}} \left| \frac{60\ \cancel{\text{s}}}{80\ \cancel{\text{g}}} \right| \frac{1000\ \cancel{\text{g}}}{1\ \text{kg}} = \boxed{-3.75\ \text{mC/kg}}$$