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ORIGINAL ARTICLE

Efficacy of a disinfectant containing silver dihydrogen citrate against GI.6 and GII.4 human norovirus

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Abstract

Aims: Human norovirus is a major public health burden and is resistant to numerous sanitizers and disinfectants. In this study, we tested the efficacy of an antimicrobial product containing a blend of silver ions and citric acid (silver dihydrogen citrate; SDC) against GI.6 and GII.4 HuNoV.
Methods and Results: Pure® hard surface disinfectant (Pure Bioscience, El Cajon, CA) was evaluated using ASTM International virucidal suspension and stainless steel carrier assays. The effect of SDC (or citrate alone) exposure on viral integrity was evaluated using RT-qPCR, transmission electron microscopy, sodium dodecyl sulphate-polyacrylamide gel electrophoresis/Western blot analysis and a receptor-binding assay. Suspension assays showed a 4·0 $\log_{10}$ reduction in RNA copy number within 5 min, while carrier assays showed a 2·0–3·0 $\log_{10}$ reduction in 30 min. Incorporating a simulated soil load into the sample matrix significantly reduced product efficacy. Treated particles displayed deformation and aggregation, a 50% reduction in viral capsid protein band intensity, and an 80% reduction in histo-blood group antigen receptor-binding ability.
Conclusions: Our results suggest that SDC acts exclusively on the viral capsid, and shows efficacy against HuNoV when used on precleaned surfaces.
Significance and Impact of the Study: This study sheds light on the mechanisms and efficacy of a novel antimicrobial against HuNoV. Our results suggest: (i) silver ions exclusively target the viral capsid, and not the RNA genome; (ii) citrate is not crucial for HuNoV capsid deformation.

Introduction

Human norovirus is a leading cause of viral gastroenteritis worldwide (Patel *et al.* 2009). In the United States, the virus is responsible for 19–21 million illnesses annually (Hall *et al.* 2013). The economic burden associated with norovirus illness is tremendous, with some estimates suggesting that disease-associated costs range as high as $2 billion annually (Hoffmann *et al.* 2012). Symptoms include nausea, vomiting and diarrhoea. While most healthy individuals recover within 24–48 h, symptoms can become life threatening in immune-compromised populations if rehydration therapy is ignored.

Human norovirus transmission occurs through ingestion of contaminated food or water, or by direct contact with infected individuals. Environmental transmission has also been documented, and episodes of vomiting or diarrhoea can contaminate surfaces with infectious virus particles that may persist for weeks (Lopman *et al.* 2012). Human norovirus is also relatively resistant to a variety of frequently used sanitizers and disinfectants, for example, alcohol-based hand sanitizers and sodium hypochlorite at regulated concentrations (Liu *et al.* 2010; Tung *et al.* 2013), further enhancing its persistence on environmental surfaces. These unique traits, combined with a low infectious dose and the fact that infected individuals shed a large amount of virus in both faecal material and vomitus, contribute to the high number of outbreaks observed annually in environments such as cruise ships, restaurants, long-term care facilities and schools (Teunis

et al. 2008; Hall 2012). Clearly, there is a need for improved measures to reduce the environmental transmission of human norovirus in these settings.

The antimicrobial properties of silver ions have been recognized for many years (Palza 2015). Silver ions are capable of disrupting cellular membranes and altering protein structure, and so they are highly effective against a broad range of micro-organisms. Unfortunately, the antimicrobial efficacy of silver ions is often short-lived, as they are highly reactive and unstable in solution. Silver ions can be stabilized in the presence of citric acid, as is the case with the patented molecule silver dihydrogen citrate (SDC; Pure Bioscience, El Cajon, CA). SDC is a blend of electrolytically generated silver ions (0·003%) in citric acid (4·846%), and has been shown to have antimicrobial activity against bacteria such as *Listeria monocytogenes* (Masuku *et al.* 2014). Evidence of antiviral effects of SDC in the literature is scarce, and, to our knowledge, only one previous study has examined the impact of SDC against human norovirus (Koromyslova *et al.* 2015). This study was observational in nature and did not include any functional assays (e.g. RT-qPCR) to estimate viral titre after SDC exposure.

Given the knowledge gap around the efficacy of SDC-containing products against nonenveloped viruses like human norovirus, the purpose of this study was to characterize the efficacy of an antimicrobial product containing SDC as an active ingredient against two human norovirus strains (GI.6 and GII.4) using both suspension and carrier assays according to ASTM International standards. A battery of tests were also performed in order to assess the effects of SDC on both the virus genome and capsid, including RT-qPCR, transmission electron microscopy (TEM), sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), Western blot and a histo-blood group antigen (HBGA) receptor-binding assay.

Materials and methods

Viruses and virus-like particles

Human faecal specimens positive for norovirus strains GI.6 and GII.4 were kindly provided by S.R. Greene (North Carolina Division of Public Health, Raleigh, NC). These clinical samples were obtained during outbreaks and were confirmed for human norovirus presence by RT-qPCR. Stool samples were prepared as 20% suspensions in phosphate-buffered saline (PBS; pH 7·2) and clarified by low-speed centrifugation (3100 ***g***) for 15 min. After centrifugation, the supernatant was split into 1-ml aliquots and stored at −80°C until use. Human norovirus GII.4 Sydney and GII.2 Snow Mountain virus-like particles (VLPs) were kindly provided by R. Atmar (Baylor College of Medicine, Houston, TX). Stock VLP concentrations were 3300 and 4300 ng μl^{-1} for GII.4 Sydney and GII.2 Snow Mountain respectively. Average size of VLPs was observed to be 30–38 nm for both strains.

Disinfection of human norovirus by silver dihydrogen citrate in suspension

Suspension assays for disinfection efficacy were performed in accordance with ASTM standard E 1052-96 (ASTM International 2011a). Briefly, a 10 μl volume of undiluted human norovirus 20% faecal suspension was mixed with 90 μl of SDC (in the form of Pure® Hard Surface disinfectant (Pure Bioscience)) for various contact times (15, 30, 60, 120, 300, 600 and 1800 s). At each sampling time point, 20 μl of this solution was added to 180 μl of 10% solution of Dey/Engley (D/E) neutralizing broth (Thermo Fisher Scientific, Waltham, MA). Samples were aliquoted and frozen at −80°C prior to nucleic acid extraction. Consistent with recommendations in ASTM standard E1052-96, virus, neutralization and cytotoxicity controls were included in all experiments.

Disinfection by silver dihydrogen citrate on human norovirus-inoculated stainless steel carriers

Stainless steel carrier assays for disinfection efficacy were performed in accordance with ASTM standard E1053-11 (ASTM International 2011b) with minor modifications (i.e. lower sample volumes, stainless steel carriers instead of glass and vortexing as a means to remove virus film instead of scraping). Briefly, a 25 μl volume of undiluted 20% faecal suspension was inoculated onto sterile stainless steel embossing tape cut into 5 × 1·5 cm carriers (DYMO Corporation, Berkeley, CA) and allowed to dry in a biosafety cabinet. After drying (approximately 2 h), 200 μl of SDC was pipetted onto inoculated strips for various contact times (15, 30, 60, 120, 300, 600 and 1800 s). At each sampling time point, the strips were aseptically placed into sterile 15-ml conical tubes containing 1·8 ml of a 10% solution of D/E neutralizing broth and vortexed vigorously for 60 s to elute the viruses and neutralize the SDC. After vortexing, the eluted samples were split into aliquots and frozen at −80°C until nucleic acid extraction. Consistent with recommendations in ASTM standard E1053-11, virus, neutralization and cytotoxicity controls were included in all experiments.

Simulated soil load conditions

All suspension and carrier assays were performed with and without an added soil load. Virus stocks in

experiments involving a simulated soil load were prepared in accordance to ASTM standard E1053-11. Briefly, a 340 μl volume of virus suspension was mixed with 35 μl of 5% tryptone (BD Difco, Franklin Lakes, NJ), 25 μl of 5% bovine serum albumin (BD Difco) and 100 μl of 0·4% mucin (type 1) from bovine submaxillary glands (Sigma), all in sterile PBS (pH 7·2). This achieved a virus stock solution having a total protein content roughly equal to that of 5% fetal bovine serum (ASTM International 2011b).

RNase treatment, nucleic acid extraction and RT-qPCR

Control and treated samples were analysed by RT-qPCR both with and without a prior RNase treatment. For samples not subjected to RNase treatment, a 100-μl aliquot of the eluate was subjected to RNA extraction using the automated NucliSENS® easyMag® system (bioMérieux, St Louis, MO) as per manufacturer instructions, with reconstitution of the final pellet in 40 μl of proprietary buffer. For pretreated samples, the 100-μl eluate aliquot was mixed with 1 μl RNase I (Promega, Madison, WI) and 11 μl 10× reaction buffer followed by incubation at 37°C for 15 min. To stop the reaction, the samples were placed on ice and immediately subjected to nucleic acid extraction. All RNA extracts were stored at −80°C until analysis by RT-qPCR.

RT-qPCR amplification targeted the conserved region of the ORF1-ORF2 junction of the human norovirus genome. For GI.6, the primers COG1F (5′-CGYTGGA TGCGNTTYCATGA-3′) and COG1R (5′-CAAGAGTC AATGTTTAGGTGGATGAG-3′) and the probes Ring1(a) (5′-FAM-AGATYGCGATCYCCTGTCCA-BHQ-3′) and Ring1(b) (5′-FAMAGATCGCGGTCTCCTGTCCA-BHQ-3′) were used (Kageyama *et al.* 2003). For GII.4, the primers JJV2F (5′-CAAGAGTCAATGTTTAGGTGGATGAG-3′) and COG2R (5′-TCGACGCCATCTTCATTCACA-3′) and the probe RING2-P (5′-FAM-TGGGAGGGCGATCGCAA TCT-BHQ-3′) were used (Jothikumar *et al.* 2005). The 25 μl RT-qPCR reaction mixture consisted of 2·5 μl of viral RNA extract, 200 nmol l^{-1} of forward and reverse primers, 200 nmol l^{-1} of fluorescently labelled TaqMan probe, 1× Bio-Rad PCR reaction buffer (Bio-Rad, Hercules, CA) and 0·5 μl Bio-Rad iScript RT mix. The reaction mixture was subjected to a one-step thermal cycling profile using a CFX96 Touch™Real Time PCR Detection System (Bio-Rad) under the following amplification conditions: (i) reverse transcription for 10 min at 50°C, (ii) initial denaturation for 5 min at 95°C and (iii) 45 cycles of 15 s at 95°C and 30 s at 55°C.

Estimation of genomic copy number was performed by comparison with a standard curve generated using RNA transcripts of amplified human norovirus genome fragments as previously performed (Manuel *et al.* 2015). These transcripts corresponded to nucleotides 5288–5615 (GI.6 strain; NCBI accession JQ388274) or 5003–5473 (GII.4 strain; NCBI accession JX126913). Stock transcript concentrations ranged from 5·33 to 6·37 × 10^{15} RNA copies per μl. The stock was serially diluted in sterile reagent grade water prior to use in the production of the standard curves using RT-qPCR. The $\log_{10}$-transformed RNA copy number was plotted against the threshold cycle (C_T) value and analysed by linear regression to make the standard curve.

Transmission electron microscopy

Because of the need for high assay resolution, TEM experiments were performed using VLPs. One microlitre of undiluted GII.2 Snow Mountain VLP stock was suspended in 9 μl SDC for a total contact time of 30 min. Untreated control samples consisted of GII.2 VLPs suspended in sterile cell culture grade water. The entire 10 μl mixture was then fixed onto carbon-coated nickel grids (Ladd Research, Williston, VT) and negatively stained with 2% uranyl acetate. The VLPs were visualized using a JEOL 1210 transmission electron microscope (TEM; JEOL-USA, Inc., Peabody, MA) at 80 kV at the Center for Electron Microscopy (North Carolina State University, Raleigh, NC).

Histo-blood group antigen-binding assay

For HBGA-binding experiments, 10 μl of a 1 : 1 diluted GII.4 Sydney VLP stock (concentration 1650 ng μl^{-1}) was suspended in 90 μl SDC or 9 μl of a 250 mmol l^{-1} stock solution of citrate buffer (pH ~2·0) for 15 s to 30 min. At various sampling time points, 10 μl of the mixture was removed and suspended in 90 μl of 10% D/E broth. Neutralized VLPs were kept on ice until use in the binding assay.

In preparation for assay, neutravidin-coated clear 96-well plate strips precoated with SuperBlock (Cat No. 15127; Thermo Fisher Scientific) were washed three times with 200 μl of PBS containing 0·05% Tween 20 (PBST). One hundred microlitres of 10 μg ml^{-1} biotinylated HBGA Type H (Cat No. 01-019; Glycotech, Gaithersburg, MD) suspended in PBS was applied to the wells and the plates incubated at 4°C overnight with gentle shaking. Wells were then washed three times with PBST. All assay steps thereafter were interspersed by three washes using PBST and performed at room temperature (RT) with gentle shaking and an incubation period of 30 min. First, 100 μl of the treated and neutralized GII.4 Sydney VLPs (and appropriate controls) was applied to each well. This was followed by 100 μl of mouse monoclonal

anti-norovirus antibody NS14 (provided by R. Atmar, Baylor College of Medicine) at a concentration of 0·27 μg ml^{-1} diluted in 5% (w/v) bovine serum albumin-PBST (BSA-PBST), and then 100 μl of goat anti-mouse horseradish peroxidase antibody (no. 62-6520; Invitrogen, Waltham, MA) diluted to 0·20 μg ml^{-1} in BSA-PBST. The 3,3′,5,5′-tetramethylbenzidine microwell peroxidase substrate system (KPL, Gaithersburg, MD) was used for colour development. Absorbance was read at 450 nm using a Tecan Infinite M200-pro microplate reader (Tecan Group Ltd, Mannedorf, Switzerland). Two wells were tested per treatment for each plate, and a total of three replicates were performed. The absorbance of negative control wells (neutralized treatment solutions with no VLP) was subtracted from all other well absorbance values to produce a normalized absorbance reading. Data were then expressed as a percentage of positive neutralization control absorbance signal.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Prior to performing SDS-PAGE experiments, GII.2 Snow Mountain VLP stock solution was diluted 1 : 1 in PBS (pH 7·2), resulting in a VLP working stock solution of 2150 ng μl^{-1}. Four microlitres of this stock was then placed in a suspension with 36 μl of SDC for 1, 5, 10 and 30 min. At each time point, 10 μl of the mixture was removed and placed into 90 μl of a 10% solution of D/E neutralizing broth. The entire 100 μl of neutralized VLP solution was then combined with 100 μl of 2X prepared Laemmli buffer (Bio-Rad) and boiled at 95°C for 5 min. Twenty-five microlitres of the boiled sample was separated using 12% Mini-Protean® TGX precast gels (Bio-Rad) with a Spectra broad range protein ladder (Thermo Fisher Scientific). Gels were stained with AcquaStain Protein Gel Stain (Bulldog Bio, Portsmouth, NH) and imaged using an AlphaImager Gel Documentation system (Protein Simple, Santa Clara, CA). Band intensities for each time point were determined using ImageJ software (National Institutes of Health; http://imagej.nih.gov/ij/).

Western blotting

The SDS-PAGE protein gels were transferred to 0·45-μm nitrocellulose membranes (Bio-Rad) using a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad) and blocked in SuperBlock blocking buffer (Thermo Fisher Scientific) overnight at 4°C. The membrane was treated with 10 ml of PBST to which had been added 15 μg of mouse monoclonal anti-norovirus antibody NS14. Membranes were then incubated at RT for 1 h with shaking, washed three times with PBST for 10 min and exposed to goat anti-mouse IgG alkaline phosphatase-conjugated antibody (Sigma) diluted 1 : 5000 in 5% skim milk-PBST for 1·5 h at RT. After three more PBST washes, membranes were developed for 10–15 min at RT with 5-bromo-4-chloro-3indoyl-phosphate/p-nitro-blue tetrazolium chloride (BCIP/NBT) solution (MP Biomedicals, Santa Ana, CA).

Statistical analysis

All experiments were replicated in triplicate. Statistical analysis was performed by one-way multiple comparisons using SAS 9·2 (SAS Institute, Cary, NC) with Tukey's HSD being used for separation of means. A *P* value of <0·05 was considered statistically significant.

Results

Efficacy of SDC against GI.6 and GII.4 human norovirus in suspension and on inoculated carriers

Figures 1 and 2 show suspension and carrier assay results, both with and without soil load respectively. In all experiments, samples without an RNase pretreatment did not show a significant reduction in human norovirus RNA copy number throughout the 30-min exposure time, suggesting that SDC works exclusively on the viral capsid. Given this observation, we focused on performing statistical analysis only on samples pretreated with RNase prior to RT-qPCR.

In a suspension assay format and in the absence of a soil load, treatment with SDC resulted in the reduction of RNA copy number for both GI.6 and GII.4 human norovirus of about 4·0 $\log_{10}$ after a 5-min exposure period(Fig. 1a,c). This number represents the assay limit of detection (LOD) for GI.6, but not for GII.4 samples (LOD of ~6·0 $\log_{10}$). Extending the time past 5 min did not yield any additional reduction in viral titre. When a soil load was incorporated into the suspension, the efficacy of SDC against human norovirus was diminished in a time-dependent manner. A maximum RNA copy number reduction of ~2·5 $\log_{10}$ was observed for both GI.6 and GII.4 samples (Fig. 1b,d). In the presence of soil load, GI.6 norovirus was more resistant to SDC than was GII.4, as 30 min was required to achieve the maximum reduction for GI.6, and only 10 min was required for GII.4 (Fig. 1b,d).

Inoculating norovirus samples onto stainless steel carriers prior to exposure to SDC (surface assays) had the effect of diminishing antiviral efficacy as compared to the suspension assay (Fig. 2). Specifically, a 30-min exposure to SDC resulted in a maximum RNA copy number

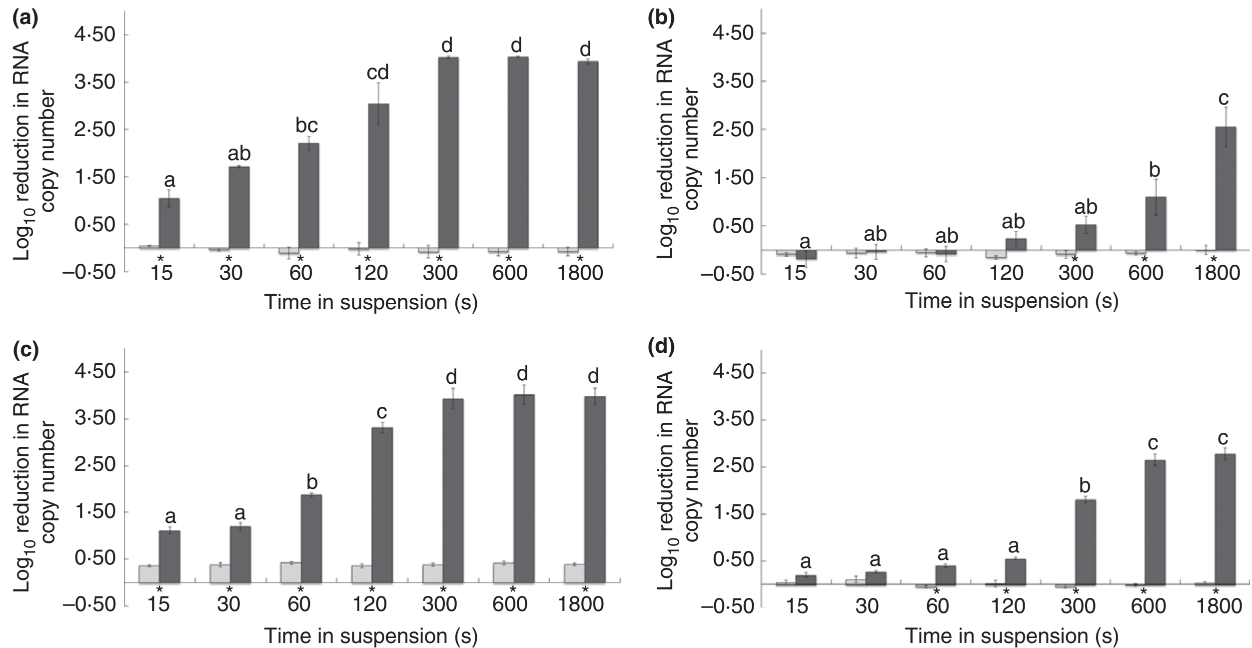

Figure 1 Effects of silver dihydrogen citrate (SDC) in suspension with human norovirus strains GI.6 and GII.4. Inactivation of human norovirus GI.6 (a and b) and GII.4 (c and d) by SDC as evaluated by RT-qPCR using suspension assay format. Clarified 20% faecal suspensions positive for either GI.6 or GII.4 human norovirus were exposed to SDC-containing disinfectant, with (b and d) and without (a and c) additional soil load for 15 s to 30 min, followed by neutralization. The samples were extracted for RNA and analysed by RT-qPCR with an RNase pretreatment (■) and without an RNase pretreatment (□). Human norovirus RNA copy number was estimated by extrapolation to a standard curve. Letters above bars indicate statistically significant differences ($P < 0.05$) between time points for samples pretreated with RNase prior to RT-qPCR. Asterisks under bars indicate instances where statistically significant differences ($P < 0.05$) were observed between samples with and without RNase pretreatment. Error bars represent standard error of the mean. All experiments were performed in triplicate.

reduction of about 2·0 and 3·0 $\log_{10}$ for GI.6 and GII.4 samples respectively (Fig. 2a,c). Again, inactivation was time dependent. When a 5% soil load was incorporated in these surface assays, no statistically significant reduction in RNA copy number was observed for either virus at all time points (Fig. 2b,d), indicating SDC to be relatively ineffective at inactivation of human norovirus on surfaces having a high degree of organic material.

Impact of SDC exposure on the capsid of human norovirus VLPs

The impact of SDC exposure on capsid integrity was further investigated using a combination of TEM, SDS-PAGE and HBGA receptor-binding assay. When visualized by TEM, GII.2 Snow Mountain VLPs exposed to SDC for 30 min displayed extensive deformation and particle aggregation as compared to control (untreated) samples (Fig. 3). Specifically, particles appeared to lose their defined centre and circular appearance when exposed to SDC.

Consistent with others (Lou *et al.* 2012), when untreated GII.2 Snow Mountain VLPs were analysed by SDS-PAGE, two protein bands with molecular masses of ~58 and ~45 kDa, corresponding with the full-length VP1 and the cleaved form of VP1 (cVP1), were observed (Fig. 4). Exposure to a 250 mmol l^{-1} solution of citrate (a major component of Pure hard surface disinfectant) did not appear to reduce human norovirus VP1 SDS-PAGE band intensity as compared to control samples, regardless of exposure time (Fig. 4a). Similar experiments with SDC showed a gradual reduction in human norovirus VP1 band intensity over time (Fig. 4b), suggesting that the specific formulation of the SDC provided the antiviral efficacy. It is likely that the silver ions are responsible for this activity. Parallel Western blotting failed to show an observable reduction in band intensity during the 30-min exposure to SDC, suggesting VLPs retain antigenic properties even after SDC exposure.

Receptor-binding assays using synthetic HBGA type H and GII.4 Sydney VLPs were used as an indirect measure of virus infectivity after exposure to SDC. Within 15 s of exposure, the affinity of VLPs for HBGA dropped to approximately 70% of that observed for the untreated VLP control sample (Fig. 5). By 10 min, this value dropped to approximately 40%, and by 30 min, the value

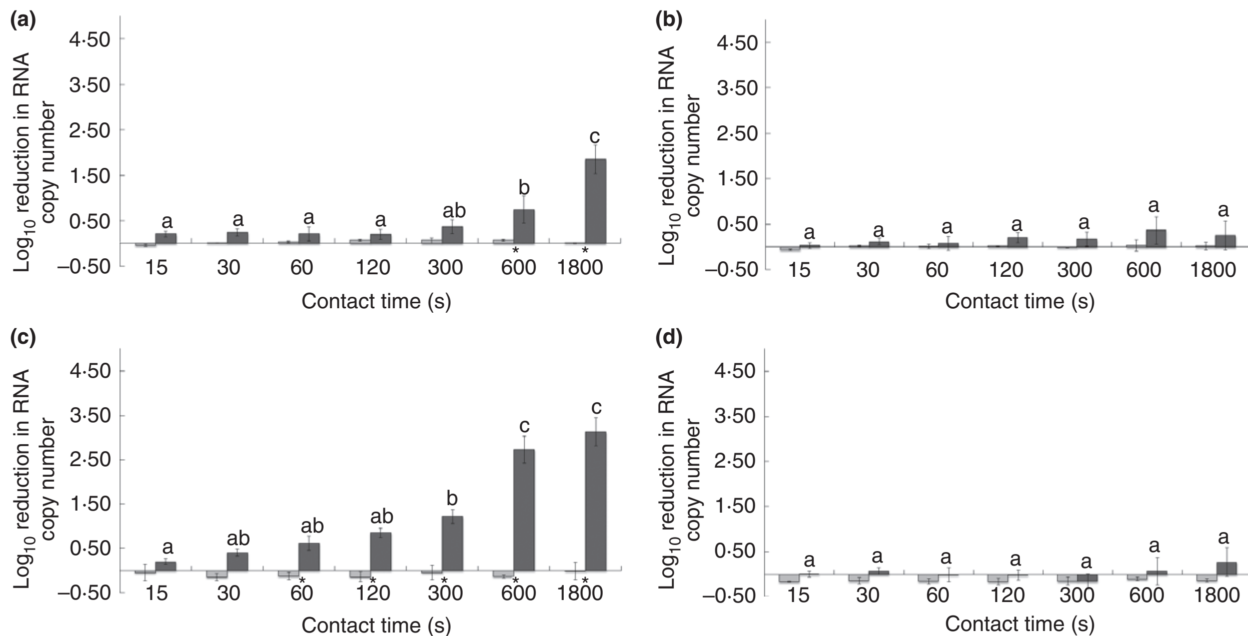

Figure 2 Effects of silver dihydrogen citrate (SDC) against GI.6 and GII.4 human norovirus samples dried onto stainless steel surfaces. Inactivation of human norovirus GI.6 (panels a and b) and GII.4 (panels c and d) by SDC as evaluated by RT-qPCR using carrier test. Clarified 20% faecal suspensions positive for either GI.6 or GII.4 human norovirus were placed onto sterile stainless steel carriers, allowed to dry and exposed to SDC-containing disinfectant, with (b and d) and without (a and c) additional soil load for 15 s to 30 min, followed by neutralization. The samples were extracted for RNA and analysed by RT-qPCR with an RNase pretreatment (■) and without an RNase pretreatment (□). Human norovirus RNA copy number was estimated by extrapolation to a standard curve. Letters above bars indicate statistically significant differences ($P < 0{\cdot}05$) between time points for samples pretreated with RNase prior to RT-qPCR. Asterisks under bars indicate instances where statistically significant differences ($P < 0{\cdot}05$) were observed between samples with and without RNase pretreatment. Error bars represent standard error of the mean. All experiments were performed in triplicate.

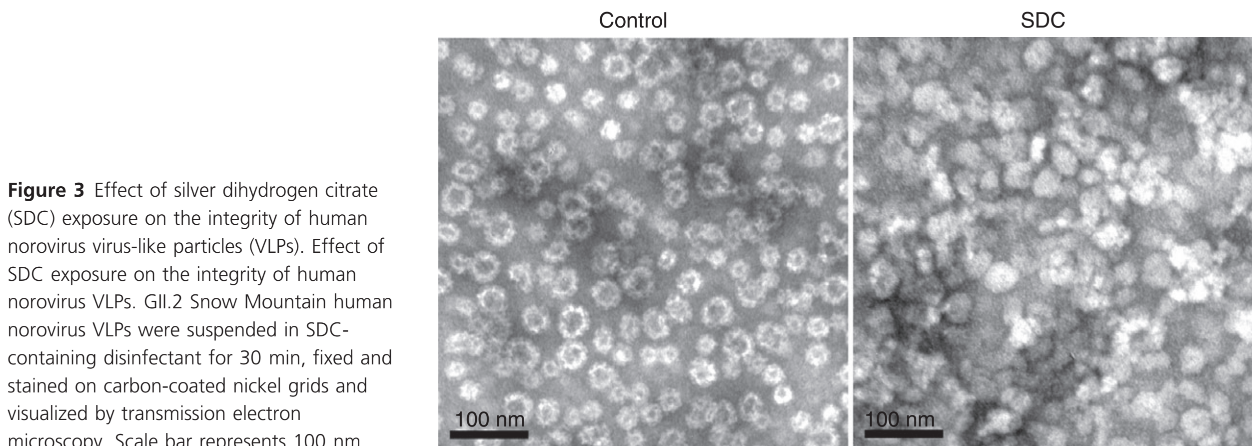

Figure 3 Effect of silver dihydrogen citrate (SDC) exposure on the integrity of human norovirus virus-like particles (VLPs). Effect of SDC exposure on the integrity of human norovirus VLPs. GII.2 Snow Mountain human norovirus VLPs were suspended in SDC-containing disinfectant for 30 min, fixed and stained on carbon-coated nickel grids and visualized by transmission electron microscopy. Scale bar represents 100 nm.

dropped to approximately 20%. In contrast, samples exposed to citrate only dropped to approximately 80% of control after the full 30-min exposure. These data suggest that exposure to SDC results in loss of HBGA-binding ability of VLPs and may be attributed to the presence of silver ions, although complete loss of binding did not occur even at the longest (30 min) exposure time point.

Discussion

In this study, the efficacy of a novel antimicrobial containing the active ingredient SDC was studied against human norovirus strains GI.6 and GII.4, as well as select VLPs. Under pristine conditions, a 4·0 $\log_{10}$ reduction in RNA copy number was observed within 5 min of SDC

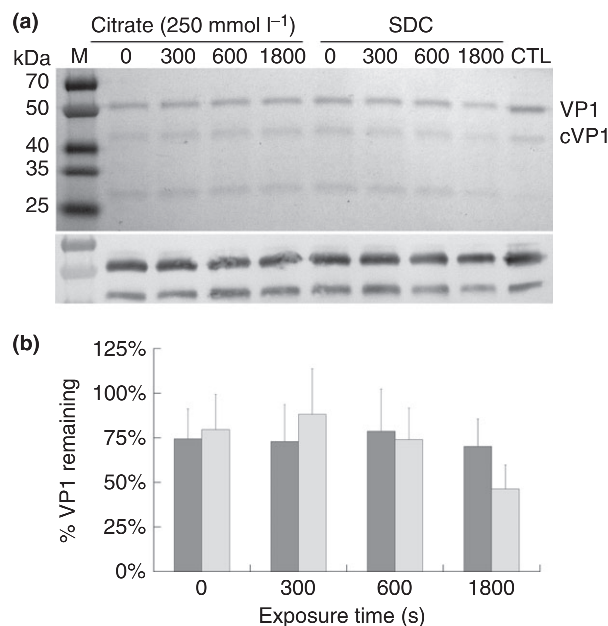

Figure 4 Impact of silver dihydrogen citrate (SDC) or citrate alone on human norovirus capsid protein as measured by SDS-PAGE and Western blotting. Impact of a 250 mmol l^{-1} citrate solution or SDC in suspension against human norovirus capsid proteins. GII.2 Snow Mountain VLPs were exposed to SDC-containing disinfectant for 5–30 min, neutralized and analysed by SDS-PAGE and Western blot using mouse monoclonal antinorovirus antibody. (a) SDS-PAGE (top) and Western blot (bottom); VP1, native full-length capsid protein; cVP1, cleaved VP1. (b) Densitometric analysis of remaining VP1 proteins detected by SDS-PAGE after 250 mmol l^{-1} citrate (■) or SDC (■) exposure.

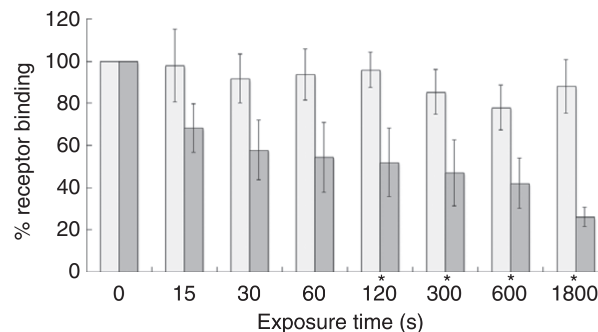

Figure 5 Impact of silver dihydrogen citrate (SDC) or citrate alone on human norovirus virus-like particle (VLP) receptor binding. Impact of SDC or citrate alone on human norovirus VLP receptor binding. Human norovirus GII.4 Sydney VLPs were exposed to either a 250 mmol l^{-1} citrate solution (■) or SDC (■) for 15 s to 30 min., neutralized and tested using the HBGA receptor-binding assay. Data are presented as a percentage of the absorbance of untreated VLPs. All experiments were performed in triplicate. Asterisks under bars indicate instances where statistically significant differences ($P < 0{\cdot}05$) were observed between SDC and citrate treated samples at each time point.

exposure for both virus strains. This degree of inactivation would meet the U.S. Environmental Protection Agency (EPA) standards for antinorovirus efficacy (4·0 $\log_{10}$ reduction in viral titre (by RT-qPCR) in 5 min in suspension) (US EPA, 2015). When 5% soil load was present, SDC was less effective at inactivating human norovirus, providing an approximate 2·5 $\log_{10}$ reduction in RNA copy number after a 30-min exposure.

The ability of organic material to diminish disinfectant efficacy is well recognized. For example, Chiu *et al.* (2015), using an antimicrobial product containing a blend of hydrogen peroxide and surfactants (accelerated hydrogen peroxide (AHP)) in suspension against human norovirus surrogates (i.e. murine norovirus and feline calicivirus), observed a significant reduction in AHP antiviral efficacy in the presence of soil load. Virto *et al.* (2005) subjected a variety of bacterial pathogens (i.e. *L. monocytogenes*, *Yersinia enterocolitica* and *Escherichia coli*) to free chlorine in the absence and presence of 150 ppm of organic matter (trypticase soy broth), finding that organic material drastically reduced the efficacy of chlorination. In fact the U.S. Centers for Disease Control (CDC) recommends the use of 1000 ppm-free chlorine for disinfecting clean surfaces contaminated with human norovirus, but up to 5000 ppm for soiled surfaces (CDC, 2011). The impact of organic material on chlorination efficacy has been attributed to the higher chlorine demand of organic compounds, which can result in a rapid decline in the available free chlorine in a solution (Virto *et al.* 2005). Silver ions have been shown to interact with thiol-containing groups (Liau *et al.* 1997), including the amino acid cysteine. Thus, the presence of organic material likely diminishes the antiviral effects of SDC by quenching silver ions, making them unavailable to react with amino acids on the human norovirus capsid.

Reduced antiviral efficacy was also observed when SDC was applied to virus-inoculated stainless steel carriers (Fig. 2). Specifically, without added soil, applying SDC to inoculated carriers resulted in only a 2·0 and 3·0 $\log_{10}$ reduction in RNA copy number for GI.6 and GII.4 strains, respectively, after a 30-min exposure. This is juxtaposed to the 4·0 $\log_{10}$ reduction in 5 min by suspension assay. The ability of SDC to inactivate norovirus inoculated onto stainless steel carriers was completely lost in the presence of 5% soil load. This discrepancy between suspension and surface tests has been previously reported (Park *et al.* 2007). Some authors have hypothesized that reduced disinfection efficacy on surfaces is due to a reduced accessibility of the virus to the disinfectant (Park *et al.* 2007). In addition, norovirus particles have been shown to partially aggregate when dried onto stainless

steel surfaces (Manuel *et al.* 2015), and it is possible that the aggregation by drying provides a protective effect by occluding available target sites on the capsid. Given that carrier assays provide more conservative estimates of virus inactivation, and are more indicative of 'real-world' situations in the sense that material is deposited onto a surface prior to testing, they could be considered a more realistic approach to evaluating surface disinfectant efficacy.

While RT-qPCR signal measures viral genome integrity, it alone is not a reliable means to quantify the presence of infectious virus. This is because viral RNA can remain amplifiable even after the capsid has been destroyed, meaning that RT-qPCR can underestimate the effectiveness of a disinfectant that works primarily by means of capsid disruption. The RNase enzyme degrades naked viral RNA and that derived from damaged capsids, preventing these genomes from being amplified. Hence, pretreating samples with RNase before performing RT-qPCR is thought to provide a more accurate estimate of the numbers of infectious virions present (Nuanualsuwan and Cliver 2002; Manuel *et al.* 2015). The lack of any observable reduction in RT-qPCR signal in the absence of RNase pretreatment in all of our experiments strongly suggests that SDC works exclusively on the viral capsid. This is supported by the TEM results, which confirmed that a 30-min exposure to SDC resulted in notable morphological changes to norovirus VLPs, similar to observations after treatment with heat, high pressure and copper (Lou *et al.* 2012; Escudero-Abarca *et al.* 2014; Manuel *et al.* 2015). Also after a 30-min exposure to SDC, SDS-PAGE/Western blot analysis showed an approximate 50% reduction in VP1 protein concentration, and the ability of norovirus VLPs to bind to HBGA receptor moieties dropped to approximately 20% of that of untreated VLPs. Collectively, the RNase-RT-qPCR, TEM, SDS-PAGE and HBGA-binding results strongly support that the capsid of human norovirus is negatively impacted by SDC exposure, most likely due to the deformation of the viral capsid protein.

Despite the well-recognized antimicrobial effects of silver, the actual active component in SDC remains debatable. Recently, Koromyslova *et al.* (2015) investigated the impact of Pure Green 24 (Pure Green LLC, Centre Island, NY), which uses SDC as its active ingredient, and citrate alone against human norovirus VLPs. Using X-ray crystallography, the authors failed to identify any conclusive effects from the silver ions on VLP structure. However, they did observe that citrate drastically altered particle morphology and impacted the ability of VLPs to bind at the HBGA-binding pocket (Koromyslova *et al.* 2015). These observations led the authors to conclude that citrate is a potential antiviral compound. Inspired by this, we examined the independent effect of SDC or citrate alone on VLPs, experiments in which we demonstrated that citrate gave a maximum reduction in capsid protein of only 25%, suggesting that silver ions seem to be crucial for the reduction in capsid protein levels (Fig. 4). Although our study was not designed specifically to compare citrate to silver ions, such a study would be warranted in the future.

In conclusion, this study demonstrates that the SDC disinfectant formulation tested was quite effective in solution, but demonstrated significantly reduced efficacy in the presence of 5% soil load or when applied directly to stainless steel-immobilized human norovirus, with lower total $\log_{10}$ reduction in genome copy number and the need for increased contact time. When applied to disinfect surfaces in the presence of 5% organic matter, the product completely lost its efficacy. The combined data from SDS-PAGE/Western blot and receptor-binding assay suggest that the disinfectant had significant impact on the viral capsid protein after a contact time of 30 min. Given these findings, surface disinfectants for which SDC is the active ingredient might best be used for applications to precleaned surfaces to maximize anti-noroviral activity. Of course, formulation matters and other SDC-containing disinfectants may perform differently. Nonetheless, SDC is potentially an environmentally friendly alternative to 'harsher' disinfectants and merits consideration for use as part of a comprehensive programme designed to control human norovirus transmission in the environment.

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Conflict of Interest

No conflicts of interest declared.

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