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RESEARCH

Surface Inactivation of a SARS-CoV-2 Surrogate with Hypochlorous Acid is Impacted by Surface Type, Contact Time, Inoculum Matrix, and Concentration

Allyson N. Hamilton[1] · Sahaana Chandran[1] · Christopher A. Baker[1,2] · Kristen E. Gibson[1]

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Abstract
Indirect contact with contaminated surfaces is a potential transmission route for COVID-19. Therefore, it is necessary to investigate convenient and inexpensive surface sanitization methods, such as HOCl, against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). The SARS-CoV-2 surrogate, Phi6 (~ 7 log PFU/mL), was prepared in artificial saliva and tripartite matrices, spot inoculated on coupons of either stainless steel or vinyl, and allowed to dry. The coupons were sprayed with either 500 ppm or 1000 ppm HOCl, and remained on the surface for 0 s (control), 5 s, 30 s, or 60 s. Samples were enumerated via the double agar overlay assay. Statistical analysis was completed in R using a generalized linear model with Quasipoisson error approximations. Time, concentration, surface type, and inoculum matrix were all significant contributors to log reduction at $P = 0.05$. Significant three-way interactions were observed for 1000 ppm, vinyl, and 60 s ($P = 0.03$) and 1000 ppm, tripartite, and 60 s ($P = 0.0121$). A significant two-way interaction between vinyl and 60 s was also observed ($P = 0.0168$). Overall, increased HOCl concentration and exposure time led to increased Phi6 reduction. Notably, the highest estimated mean log reduction was 3.31 (95% CI 3.14, 3.49) for stainless steel at 60 s and 1000 ppm HOCl in artificial saliva, indicating that this method of sanitization may not adequately reduce enveloped viruses to below infective thresholds.

Keywords Fomite · Phi6 · Hypochlorous acid · Vinyl · Stainless steel

Introduction

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic continues to cause illness and death throughout the world, with nearly 6.5 billion illnesses and more than 6.6 million deaths attributed thus far (Schiffman & Conlon, 2019). Patel and others note that there are six known ways in which SARS-CoV-2 can be spread (respiratory/droplet, indirect, fecal–oral, vertical, sexual, and ocular) (Patel et al., 2020). Even with widespread illness and death, one-in-five U.S. adults are unwilling to be vaccinated against SARS-CoV-2 (Allen et al., 2021). Therefore, additional preventative measures must be implemented in order to reduce the transmission of SARS-CoV-2 and subsequent burden on healthcare systems.

Since BSL-3 environments are needed to work with live samples of SARS-CoV-2, surrogates have been widely employed. Phi6 is a member of the *Cystoviridae* family and is among the few bacteriophages to have a lipid envelope (Vidaver et al., 1973). The bacteriophage has a double-stranded 13.4 kbp RNA genome and is similar in size to SARS-CoV-2 at approximately 75 nm in diameter (Fedorenko et al., 2020; Frilander et al., 1995; Gonzalez et al., 1977). Phi6 has been investigated as a surrogate for both Ebola viruses and SARS-CoV-2 (Baker et al., 2022a; Bangiyev et al., 2021, Fedorenko et al., 2020; Gallandat et al., 2017; Whitworth et al., 2020; Wood et al., 2020;). The persistence and transfer of Phi6 on fomites has also been widely explored (Anderson & Boehm, 2021; Baker et al., 2022a, 2022b; Bangiyev et al., 2021; Fedorenko et al., 2020; Gallandat et al., 2017; Whitworth et al., 2020; Wood et al., 2020). The host of Phi6 is *Pseudomonas syringae* pathovar *phaseolicola* (Pph), making both Phi6 and its host BSL-1

✉ Kristen E. Gibson
keg005@uark.edu

[1] Department of Food Science, Center for Food Safety, University of Arkansas System Division of Agriculture, 1371 West Altheimer Dr., Fayetteville, AR 72704, USA

[2] Present Address: U.S. Food and Drug Administration, Center for Food Safety and Applied Nutrition, 5001 Campus Drive, College Park, MD 20740, USA

microorganisms simple to cultivate and use as a surrogate for highly pathogenic enveloped viruses (Aquino de Carvalho et al, 2017).

Hypochlorous acid (HOCl) is naturally produced by immune cells in mammals in response to injury or infection (Kettle & Winterboum, 1997), and is capable of viral inactivation by forming chloramines and nitrogen-centered radicals. These reactive compounds result in strand breaks of genetic material and destruction of the nucleic acids that form the virus (Block & Rowan, 2020; Winter et al., 2008). In situ production of HOCl can also be achieved by combining non-iodinated salt, water, acetic acid (e.g., vinegar), and electrolysis (Block & Rowan, 2020; Chlorking, 2021). More specifically, non-iodinated salt is mixed with water to produce a salt solution followed by the addition of vinegar (Farah & Al-Haj Ali, 2021). This solution is then subjected to electrolysis for production of HOCl, or electrolyzed water which can be acidic or neutral depending on the process.

The hypochlorite ion (OCl^-) has been tested against different viruses including three strains of murine hepatitis virus, canine coronavirus, human norovirus, murine norovirus, and MS2 bacteriophage (Dellano et al., 2009; Park et al., 2007; Saknimit et al, 1988). The typical use of sodium hypochlorite is 500 ppm, but the summarized data by Kampf et al. (2020) suggest exposure to 1000 ppm for 1 min is required for complete inactivation of SARS-CoV-2 and note that 2100 ppm for 30 s was effective as a disinfectant against murine hepatitis virus.

This study aimed to determine the efficacy of aqueous HOCl in inactivating Phi6, a surrogate for SARS-CoV-2, on surfaces frequently encountered in consumer-facing environments such as food service dining areas, waiting rooms, hospital triage areas, and in-patient rooms. Tested parameters included inoculum matrix (ASTM formulated artificial saliva and tripartite load), surface type (stainless steel and vinyl), HOCl concentration (500 ppm and 1000 ppm), and exposure time (5 s, 30 s, and 60 s).

Materials and Methods

Phi6 Production

Phi6 bacteriophage (HER102) production and *Pseudomonas syringae* pathovar *phaseolicola* (Pph) (HER1102) growth were performed in lysogeny (LC) broth [10 g/L NaCl (VWR, Radnor, PA), 10 g/L tryptone (VWR), 5 g/L yeast extract (VWR) ultrapure water, pH adjusted to 7.5] as previously described (Baker et al., 2022b). Briefly, one Pph colony was selected from an LC plate and grown overnight in 25 mL of LC broth with constant agitation. The following day, Phi6 was propagated by adding 200 µL Pph overnight stock and 100 µL of Phi6 stock (~ 10 log plaque forming units (PFU)/mL) to 5 mL of LC soft agar. The resulting mixture was poured onto an LC agar plate and distributed. This double agar overlay assay (DAL) is based on Kropinski et al. (2009). Once solidified (~ 15 min) the plates were inverted and incubated at 25 °C until a lacy appearance was present (~ 24 h). The soft agar layer was collected into 4 mL of LC broth with a 25 cm cell scraper (VWR) before vortexing and centrifuging for 10 min at 3000×*g* and 4 °C. The supernatant was filtered using a 0.45 µm sterile polyethersulfone syringe filter (Whatman, Buckinghamshire, United Kingdom) and stored at 4 °C. Phi6 stock was titered using the DAL assay.

Inoculum Preparation—Artificial Saliva and Tripartite

The artificial saliva matrix was chosen to mimic cough or sneeze ejecta and prepared according to ASTM E2721-16. In brief, the artificial saliva matrix consisted of 0.21 g/L KH_2PO_4 (Sigma-Aldrich, St. Louis, MO), 0.43 g/L K_2HPO_4 (Fisher Scientific, Loughborough, UK), 0.04 mg/L $MgCl_2{\cdot}7H_2O$ (Alfa Aesar, Ward Hill, MA), 0.11 g/L NH_4Cl (VWR), 0.12 g/L $(NH_2)_2CO$ (VWR), 0.13 g/L $CaCl_2$ (VWR), 0.19 g/L KSCN (Acros Organics, Carlsbad, CA), 0.42 g/L $NaHCO_3$ (Fisher Scientific), 0.88 g/L NaCl (VWR), 1.04 g/L KCl (VWR), and 3 g/L mucin (Sigma-Aldrich) at pH 7 (ASTM International, 2016; Owen et al., 2021). The tripartite matrix was chosen to mimic fecal material shed by infected individuals and prepared as per international standard ASTM E2197-17 (ASTM International, 2017). In brief, the tripartite matrix consisted of 0.8 g/L bovine mucin (Sigma-Aldrich), 2.5 g/L bovine serum albumin (VWR), and 3.5 g/L tryptone (VWR) (ASTM International, 2017; Kasloff et al., 2021; Riddell et al., 2020; Sattar et al., 2003). Phi6 stock was added to each matrix to obtain inoculum levels of approximately 7 log PFU/mL.

Surface Preparation and Inoculation

Coupons (5 cm × 5 cm, 25 cm^2) made of stainless steel and vinyl were prepared as previously described (Baker et al., 2022b). Briefly, the stainless steel coupons were wrapped in aluminum foil and steam-sterilized at 121 °C, 15 psi for 30 min. Vinyl coupons could not be treated in this manner due to their physical composition. Prior to transfer trials, both surface types were exposed to UV light in a biosafety cabinet (30 min). After UV treatment, 100 µL of 7 log PFU/mL Phi6 inoculum in either artificial saliva or tripartite matrix was applied to the coupons in 8 to 12 spots to simulate droplets of bodily fluids. The droplets were allowed to dry for 45 min (i.e., the time at which the inoculum was visibly dry) in a biosafety cabinet under ambient conditions.

HOCl Preparation

HOCl was generated using the ChlorKing® HYPOGEN 5.0 at a pH of 6 and HOCl concentration of either 500 ppm or 1000 ppm (Chlorking, 2021). Briefly, the HYPOGEN 5.0 applies electrochemical activation of water and salt brine to produce slightly acidic or neutral electrolyzed water. The free chlorine concentration and pH of the HOCl solution was verified via the *N*,*N*-diethyl-p-phenylenediamine (DPD) method using a free chlorine kit (Hanna Instruments, Woonsocket, RI) and pH meter (Fisher Scientific), respectively.

Surface Treatment and Elution

After drying for 45 min in a biosafety cabinet, the coupons were sprayed with HOCl three times from a 30 cm distance to emulate spray bottle application of the HOCl solution. The approximate volume per spray was 1 mL. The HOCl remained on the coupons for 5 s, 30 s, or 60 s prior to elution with 2 mL of Dey-Engley neutralizing broth by repeated pipetting (five times), after which samples were diluted and plated via the DAL assay as described for Phi6 propagation.

Statistical Analysis

All experiments were performed in technical duplicates with two experimental trials. Log reductions were calculated as $-\log_{10}\left(\frac{\text{Final PFU}}{\text{Initial PFU}}\right)$, and data were analyzed in R Studio using a generalized linear model (GLM) with Quasipoisson error distributions due to heteroscedasticity, non-normality, and overdispersion (R Studio Team, 2020). More specifically, the Q–Q plot indicated non-normality of the data based on deviation of the tails from the reference line. Regarding heteroscedasticity, the ratio between the largest and smallest fitted residual is 2748.83 indicating significant deviation from the threshold (1.50) for homoscedasticity. To quantify overdispersion, the residual deviance was divided by the residual degrees of freedom to yield a value of 53.26. Since this value is much greater than one, a Quasipoisson distribution was applied in place of a Poisson distribution. The treatment means and their associated standard errors were calculated using estimated marginal means. Statistical differences between treatments were determined using multiple pairwise comparisons and visualized using compact letter display. The data were analyzed in R (R Core Team, 2021) using the base, base, ggplot2 (Wickham, 2016), emmeans (Length et al., 2021), tidyverse (Wickham et al., 2019), ggpubr (Kassambara, 2020), gdata (Warnes et al., 2022), rstatix (Kassambara, 2021), lme4 (Bates et al., 2015), lmertest (Kuznetsova et al., 2017), multcomp (Hothorn et al., 2008), and multcompView (Graves et al., 2019) packages.

Results

All raw data are plotted in Fig. 1, which gives the measured log reductions of Phi6 against the time in seconds for which HOCl was applied to the surface at either 500 or 1000 ppm. Figure 1 is faceted to show differences between surface type and inoculum matrix. Significant three-way interactions were observed for 1000 ppm, vinyl, and 60 s ($P=0.03$) and 1000 ppm, tripartite, and 60 s ($P=0.0121$). A significant two-way interaction between vinyl and 60 s

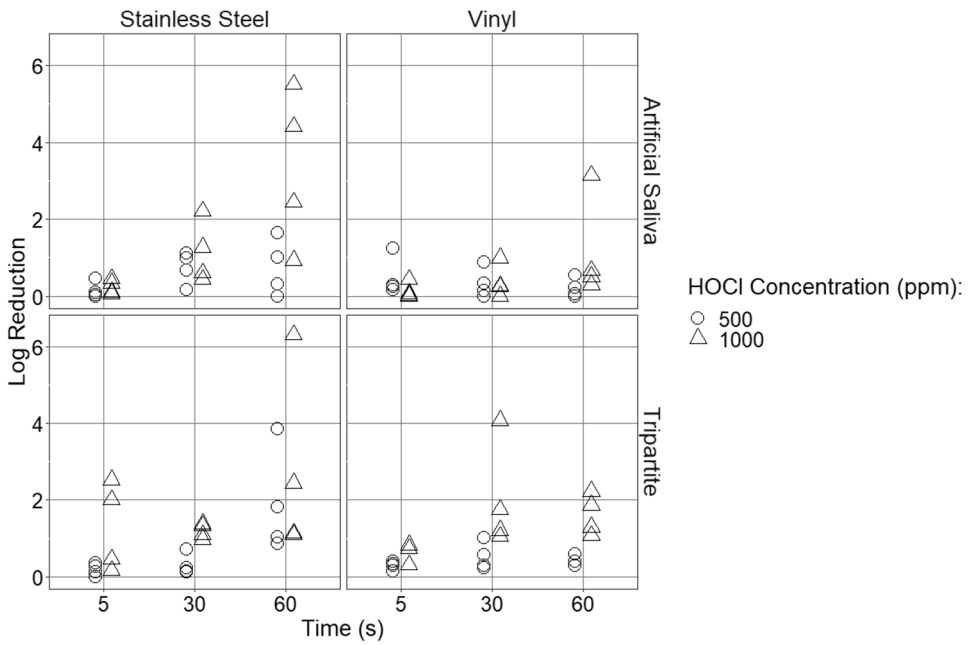

Fig. 1 Raw data of log reductions based on HOCl concentration (shape), exposure time, surface material, and inoculum matrix

was also observed ($P = 0.0168$). Due to the presence of significant interaction effects, conclusions about main effects cannot be made.

The estimated mean log reductions with their 95% confidence intervals and the statistical groupings from the post-hoc analysis are shown in Fig. 2, where it can be observed that 1000 ppm HOCl resulted in significantly more (or equal) Phi6 reduction than 500 ppm HOCl. Additionally, at constant concentration, 60 s exposure times consistently led to more Phi6 reduction than 5 s exposure times. Significant differences between stainless steel and vinyl surface types were observed for concentration, exposure time, and inoculum matrix. Notably, the highest and lowest estimated mean log reductions were 3.31 (95% CI 3.14, 3.49) for stainless steel at 60 s and 1000 ppm HOCl in artificial saliva and 0.135 (95% CI 0.11, 0.17) for vinyl at 5 s and 1000 ppm HOCl in artificial saliva.

Discussion

Past studies have explored the efficacy of OCl^- inactivation of viruses. Saknimit et al. (1988) measured log reductions of canine coronavirus and two strains of murine hepatitis virus after 10 min exposure times at both 10 ppm and 100 ppm of sodium hypochlorite (NaOCl). In agreement with the present study, higher concentrations of the hypochlorite ion led to increased reduction of all viruses, with canine coronavirus undergoing 0.9 log and 1.05 log reductions at 10 ppm and 100 ppm NaOCl, respectively. A more marked difference between concentrations was observed for the murine hepatitis virus strains: 0.41 log reductions at 10 ppm HOCl and 2.54 log reductions at 100 ppm HOCl, on average (Saknimit et al., 1988). The present study reports estimated mean log reductions of 3.04 (95% CI 2.21, 4.17) for 1000 ppm HOCl and 1.32 (95% CI 0.81, 2.13) for 500 ppm HOCl at 60 s. At 5 s, estimated mean log reductions were 0.77(95% CI 0.41, 1.44) for 1,000 ppm HOCl and 0.17 (95% CI 0.04, 0.65) for 500 ppm HOCl.

Park et al. (2007) investigated liquid- and fog-based HOCl inactivation of human norovirus and its surrogates (MS2 bacteriophage and murine norovirus) on stainless steel and ceramic coupons at three HOCl concentrations (18.8 ppm, 38 ppm, and 188 ppm) in a 1% human stool matrix. Others have also used MS2 bacteriophage as a surrogate for SARS-CoV-2 (Cadnum et al, 2020; Rockey et al., 2020). The required time for a 3 log reduction of MS2 was 5 min for stainless steel and unglazed ceramic at both 18.8 and 38 ppm HOCl. At 188 ppm HOCl, only 1 min of contact time was required to obtain a 3 log reduction of MS2 (Park et al., 2007). This reported log reduction of MS2 at 188 ppm HOCl and 60 s is similar to values observed in the present study for 1000 ppm HOCl at 60 s in tripartite [2.62 (95% CI 1.69, 4.06)] indicating that Phi6 may be a more conservative surrogate for SARS-CoV-2 than MS2 when evaluating virus inactivation by HOCl. It is worth noting that Park et al. (2007) used a 1% stool matrix while the present study used tripartite (and artificial saliva), so it is possible that the matrix composition also played a role in HOCl efficacy (Baker et al., 2022a; Bangiyev et al., 2021; Park et al., 2007).

Hatanaka et al. (2021) tested SARS-CoV-2 inactivation with HOCl in suspension tests. The authors reported that incubation with a 125 ppm HOCl solution for 10 min or a 250 ppm HOCl solution for 5 min inactivated SARS-CoV-2

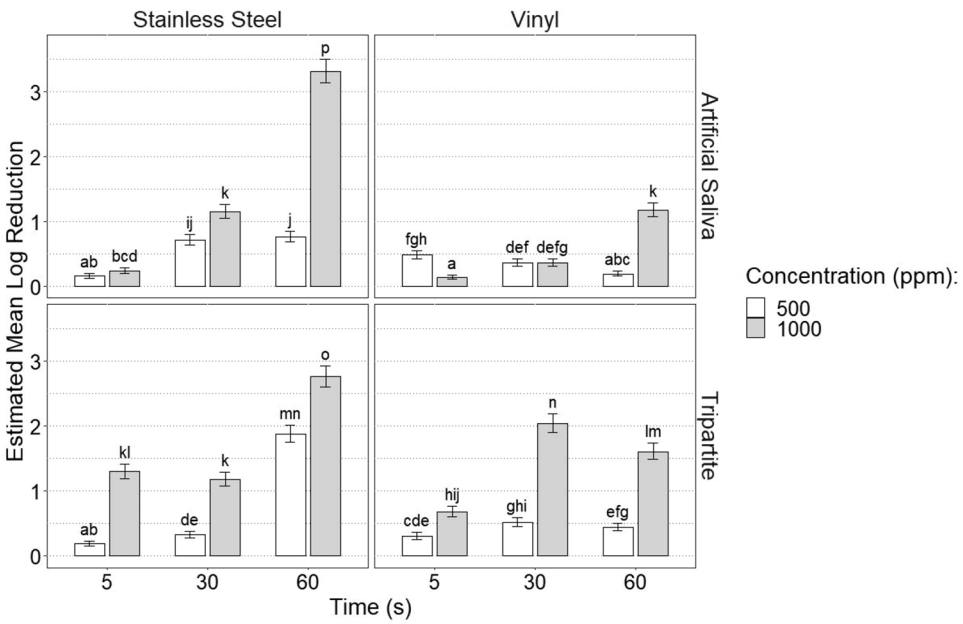

Fig. 2 Generalized Linear Model with Quasipoisson Errors for HOCl inactivation of Phi6 virus. Compact letter format is used to designate statistical differences between treatments at $P = 0.05$

by more than 4 log tissue culture infectious dose ($TCID_{50}$) per ml (Hatanaka et al., 2021). Unfortunately, the authors did not test sanitization of surfaces or within complex soils that SARS-CoV-2 would be realistically associated with. It is well-established that inactivation in suspension is often not equivalent to inactivation on a surface, especially in the presence of organic material (Lin et al., 2020). For instance, Kindermann et al. (2020) compared the efficacy of sodium hypochlorite in suspension and on carrier surfaces for inactivation of a lipid enveloped virus, bovine viral diarrhea virus (BVDV), among other viruses. The authors reported faster times to complete reduction in suspension tests (3 min) than on a stainless steel surface (5 min) for BVDV and 4500–6500 ppm free chlorine (Kindermann et al., 2020).

Between 100 and 2000 infectious viral particles of SARS-CoV-2 is adequate to cause infection, and the viral load in sputum has been measured at a maximum of 2.35×10^9 copies per mL (Karimzadeh et al., 2021; Prentiss et al., 2022). Riddell et al. (2020) demonstrated that SARS-CoV-2 remains viable for 28 days when dried onto non-porous surfaces at loads and in matrices equivalent to those excreted by humans under ambient conditions (20 °C and 50% relative humidity), but not all studies agree (Baker & Gibson, 2022; Wölfel et al., 2020). Characterization of SARS-CoV-2 persistence on surfaces is critical to understanding transfer potential and subsequent disease risk as well as developing evidence-based cleaning and disinfection practices.

Recently, Baker et al. (2022b) demonstrated bidirectional transfer of Phi6 suspended in artificial saliva and tripartite load between human skin and fomite surfaces such as touchscreen, vinyl, stainless steel, aluminum, wood, and plastic. The highest observed transfer rate from skin to surface was 22.0% (95% CI 12.8, 35.0) for touchscreen, and the highest observed transfer rate from surface to skin was 6.83% (95% CI 4.77, 9.69) for aluminum. Skin to surface transfer rates for vinyl and stainless steel were 10.9%, (95% CI 5.29, 21.3) and 2.5%, (95% CI 0.68, 9.03), respectively. Surface to skin transfer rates for vinyl and stainless steel were 4.69% (95% CI 3.03, 7.19) and 6.03% (95% CI 4.11, 8.76), respectively (Baker et al., 2022b). The low infective dose, high concentration in bodily fluids, and transferability between skin and surfaces emphasizes the importance of characterizing disinfectant efficacy and establishing proper instructions for use.

Some limitations of the present study include the use of Phi6 as a surrogate for SARS-CoV-2, utilizing HOCl at a single pH value, and testing only two surfaces. Other surfaces that could be examined in future studies include touchscreen, laminate flooring, and painted cinderblock, as these surfaces are prevalent in both healthcare and other consumer-facing environments. Additional HOCl concentrations could be evaluated, but those used in the present study were chosen based on the hypochlorous generator's manufacturer settings, as these concentrations would mimic the most likely implementation based on user feasibility. Exposure times investigated in the present study were chosen based on realistic expectations of sanitizer use. In practice, most sanitizers and disinfectants are sprayed on and immediately wiped off, and the study authors aimed to model the implications of that behavior. In the future, the impact of wiping the surface on pathogen removal should also be tested (Gibson et al., 2012). Additionally, evaluating longer contact times in the future could be useful to characterize tradeoffs between contact time and HOCl concentration.

The level of virus reduction observed in the present study indicates that HOCl—at either 500 ppm or 1000 ppm—would be unlikely to inactivate SARS-CoV-2 to a degree which would prevent human illness given the known persistence and transfer properties of the virus. Extended contact times and sanitization with wiping should be tested. Other sanitization and disinfection methods may be explored in order to protect consumers from infection.

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Declarations

Competing interests The authors have no competing interests to declare that are relevant to the content of this article.

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