Evaluating Chlorine Dioxide Gas as an Antiviral Agent: Insights from the Development, Optimization, and Application of a MS2 Bacteriophage Model System *

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ABSTRACT

Since emergence of the SARS-CoV-2 virus, the etiological agent causing COVID-19, the need to identify antiviral agents for disinfection purposes has dramatically increased. Chlorine dioxide gas has previously been identified as an antibacterial agent with strong oxidizing capabilities. Additionally, the MS2 bacteriophage was identified as a suitable surrogate for the development, evaluation, and application of virucide decontamination methods. The purpose of this study was to identify and assess the antiviral properties of chlorine dioxide gas and to identify optimum physical conditions for potential deployment in support of current antiviral disinfection needs. Using the MS2 bacteriophage model system, studies used the double-layer agar plaque assay technique to design, optimize, and evaluate the antiviral activity of chlorine dioxide gas. Results support the potential use of chlorine dioxide gas as an antiviral agent and show that environmental factors heavily influence the ability of chlorine dioxide gas to act as an antiviral agent. (1925), commenting particularly on those practices' impact on the internal worlds of Clarissa Dalloway and Septimus Smith.

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Introduction

Since their discovery in the 1800s, viruses have been of interest to the scientific community because of their potential to cause a variety of diseases in humans. Viruses have altered many aspects of people's lives throughout human history (Oldstone, 2010). For example, notable diseases caused by viruses include smallpox, influenza, COVID-19, and human immunodeficiency virus (HIV). It is estimated that nearly 300 million people were killed by smallpox in the twentieth century. In perspective, more than three times more deaths were associated with the virus than wars in the twentieth century (Oldstone, 2010). Most recently, the SARS-CoV-2 virus was responsible for the novel outbreak of COVID-19, which has claimed the lives of over five million worldwide (Johns Hopkins University, 2020). As Nobel Prize winner Peter Medawar says, "It has been well said that a virus is a bad piece of news wrapped up in protein" (Medawar and Medawar, 1985).

Viruses are submicroscopic biological agents and consist of a basic genome (either deoxyribonucleic acid or ribonucleic acid) and a protein coating. Categories of viruses are based on structural features, type of nucleic acid, and the presence or absence of an envelope. Viruses are obligate intercellular parasites, which means that viruses are reliant upon a host cell for replication (Pellett et al., 2014). Intercellular viral replication is a feature of viral infection in humans.

Viral replication can be characterized by absorption, penetration, genomic replication, reassembly, and release. The initial step for viral replication is the protein coating on the virus binding to the host cell's surface; the protein coating and the binding site on the cell are specific. The cell must then intake the virus into the cell by endocytosis, which is a packaging of the virus into a vesicle. The virus uses the host cell's environment to replicate the viral genetic information and protein coatings. Once the necessary genetic materials are created, the virus reassembles and is released from the cell, completing the replication cycle. Typically, as a result of viral replication, the host cell ruptures and dies (Cowan et al., 2019).

Because of the labor required for culture of eukaryotic cell viruses and the pathogenic nature of some viruses, bacteriophages have become an excellent model organism for virological studies (Keen, 2015). Bacteriophages are unique viral particles that only infect bacteria. Bacteriophages have similar biological features to viruses infecting human eukaryotic cells, thus, allowing bacteriophages to serve as a model organism (Clokie et al., 2011). The bacteriophage MS2, bacteriophage Phi-6, MHV, 229 E, and the SARS-CoV-2 virus were recently compared to evaluate their suitability for virological studies. In comparison, the MS2 bacteriophage offered the most promising

potential as a model organism for antiviral studies because of its ease of use and high resistance to inactivation (Ratliff and Oudejans, 2021). Additionally, MS2 bacteriophage is easily recognizable because of its well-known conjugate host, *Escherichia coli*.

For thousands of years, disinfectants have been employed to limit the spread of disease. The earliest mention of a chemical disinfectant is noted in 800 B.C. in Homer's *Odyssey*, in which sulfur was used to disinfect a room. In ancient times, sulfur, mercury, copper, acids, and salts were used for disinfection (Blancou, 1995). Although it has only been within the last 300 years, disinfectants have gained more widespread use in the scientific community. Chlorine, discovered in 1744, became one of the first industrial chemical disinfectants (Hugo, 1991). The early 1800s started with advertisements for the use of phenols for disinfection (Virox Technologies). Later, in 1839, iodine became another common disinfectant used as an antibacterial agent. Additionally, the use of hydrogen peroxide and formaldehyde became widely used by the end of the 19th-century (Hugo, 1991). The 1950s introduced the use of alcohol-based products for disinfection, and by the 1970s, use of ammonium-based products started to be used by commercial manufacturers (Virox Technologies). For almost 3,000 years, humans have employed antimicrobial agents to defend against infectious diseases.

In 1814, Sir Humphrey Davy is reported to be the first person to discover chlorine dioxide (ClO2), which is now recognized as an antibacterial agent. Davy discovered ClO2 gas by mixing sulphuric acid with potassium chlorate (Lenntech, 2021). Since the discovery, ClO2 has been used in a variety of ways.

Chlorine dioxide gas and liquid are potent oxidizing agents and have a history of different practical applications. A notable historical use was the use of ClO2 gas in the disinfection of *Bacillus anthrax* from federal buildings during the intentional release of anthrax spores in 2001 (Rastogi et al., 2010). Chlorine dioxide gas has also historically been used for the disinfection of drinking water (Aieta and Berg 1986). Additionally, ClO2 has more recently been used in the disinfection of medical equipment such as endoscopes (Isomoto et al., 2006). Although ClO2 gas and liquid have been historically used for many practical applications, generating the gas or liquid is often challenging.

Previous studies conducted on ClO2 gas at Middle Tennessee State University have aptly demonstrated the antibacterial properties of the gas. Several studies have examined the potential practical uses of ClO2 gas (Bhawana et al., 2014; Newsome et al., 2009; Stubblefield and Newsome, 2015). Additionally, studies have been aimed at evaluating the use of ClO2 to inhibit the hemagglutinin protein on the influenza virus (Gormsen, 2016). These preliminary studies place Middle Tennessee State University in a unique position to continue investigating the antimicrobial properties of ClO2.

The use of ClO2 gas has historically been restrained because of the difficulties in generating the gas. The gas is too unstable for transportation and must be prepared at the application site. Presently, this presents a problem because the gas has typically been generated using trained personnel and expensive machinery (Stubblefield and Newsome, 2015). Recently, new technology has emerged, allowing one to quickly produce ClO2 gas (Smith et al., 2014). This current study is based on use of the ICA-TriNova system, which involves mixing a solid acid and base in a gas-permeable bag to produce ClO2 gas. It does not require machinery or highly trained professionals to easily produce ClO2 gas. Additionally, the novel ICA-TriNova ClO2 production system poses minimal safety risk to the individual handling the gas at low concentrations. However, inhaling the gas at high concentrations can lead to lung irritation and toxicity, underscoring the importance of caution during its use.

Most recently, ClO2 gas has attracted the attention of scientists because of its oxidative properties that may allow ClO2 to express antiviral properties. Several studies have been aimed at evaluating the antiviral properties of ClO2 gas in different model organisms (Ogata et al. 2016; Ogata and Shibata 2008; Ogata 2012; Gormsen 2016; Sanekata et al. 2010). Although most studies published to date evaluate the inactivation of the surface portions of these viruses, they do not address the actual ability of ClO2 to inactivate the virus. Also, several of the above-cited pieces of literature require that the ClO2 gas be held at a particular part per million for a specific time for viral inactivation, which is a substantially difficult task (Kály-Kullai et al., 2020). Additionally, much of the literature in circulation has a financial interest in the study's success. Lastly, the current literature lacks in its ability to show the inactivation of non-enveloped viruses, which are known for their inability to be inactivated in the presence of antiviral agents (Lin et al. 2020). The lack of data to support the ability of ClO2 gas to inactivate viruses has prompted this current investigation.

Few studies have shown that ClO2 oxidatively interacts with amino acids, which aid in the infection process by viral particles. Specifically, ClO2 oxidatively interacts with cysteine, methionine, tyrosine, and tryptophan (Noszticzius et al., 2013). Studies have also shown that haemagglutinin and neuraminidase are inactivated in the presence of ClO2 (Ogata and Shibata, 2008; Gormsen, 2016). Most studies addressing the mode of viral inactivation by ClO2 have been investigated using enveloped viruses. In regard to enveloped viruses, one study evaluated the effects of ClO2 on viral RNA. The study revealed that the positive-sense RNA poliovirus genomic information was affected by ClO2 (Alvarez and O'Brien, 1982). Comprehensive knowledge of the mechanism of action of ClO2 against viruses remains limited and poorly understood.

The objective of this study was to develop a system by which the antiviral activity of ClO2 gas could be examined, quantified, and analyzed to aid in the investigation of potential antiviral agents (Figure 1). In optimization, factors such as humidity, exposure time to ClO2 gas, and substrates were examined. Additionally, this study was aimed at developing a system that would be easily recognizable by the Environmental Protection Agency as a valid mechanism for future studies to examine the antiviral properties of ClO2 gas. This included the use of the MS2 bacteriophage as the model organism of choice due to its high resistance to inactivation as a nonenveloped virus. Thus, studies were modeled in accordance with other Environmental Protection Agency publications and presentations (Oudejans and Ratliff, 2021; Wyrzykowska-Ceradini et al., 2019).

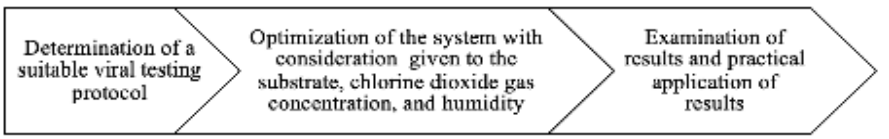

Figure 1: Research objectives flow chart

Materials and Methods

Viral and bacterial cultures used for this study consisted of the MS2 bacteriophage and *Escherichia coli* as the conjugate host. The MS2 bacteriophage (15597-B1) and *Escherichia coli* (Migula) Castellani and Chalmers (15597) were purchased from American Type Culture Collection (ATCC). Upon receipt of the MS2 bacteriophage, it was reconstituted in 2 mL of deionized water and stored at 4° C. Prior to use, bacteriophage was diluted 1:10 in sterile deionized water and stored at 4° C. Additionally, upon receipt, *Escherichia coli* was cultured, and aliquots stored (-70° C). When needed, samples were thawed, plated onto trypticase soy agar (TSA), incubated at 37° C, and stored at room temperature after 24 hours of incubation.

Trypticase soy agar was prepared by mixing 20 grams of TSA powder with 500 mL of deionized purified water, brought to a boil, autoclaved, and evenly divided into petri dishes aseptically. Trypticase soy broth (TSB) was prepared by mixing 15 grams of the powder with 500 mL of deionized purified water, brought to a boil, 4.5 mL was pipetted into test tubes, capped, and autoclaved. Nutrient agar was prepared by mixing 1 gram with 99 mL of deionized purified water, heated to boiling, and 4.0 mL were pipetted into test tubes, capped, and autoclaved. All media was stored at 4° C.

Traditionally, ClO2 gas has been generated by placing sodium chlorite in aqueous solution causing its production. Although, examination of ClO2 gas's properties and uses has been difficult due to the gas's instability (Stubblefield and Newsome 2015). Thus, investigation of ClO2 gas's antiviral properties was modeled on a solid acid base

mixture, a novel system of examination. The ICA-Trinova (Newman, GA) ClO2 gas release system was employed as the deployment mechanism of choice. This novel system allows scientists to swiftly generate ClO2 gas in a cost-effective, safe, and easy-to-use way. The system uses two granulated solid chemicals, a base (reagent A) and an acid (reagent B), mixed in a gas permeable pouch to produce ClO2 gas.

Prior studies conducted at Middle Tennessee State University with ClO2 have used an airtight gassing bucket (Gormsen, 2016). This bucket has been chosen as a suitable gassing container for the current study due to the airtight lid and internal air circulating fan to promote even distribution of the gas in the chamber. Additionally, for this conducted study, mathematical formulas have been derived by ICA-Trinova for determination of parts per million (PPM) of gas present in the container.

A Union 10' x 20" galvanized steel roll was purchased from Lowes and used for the metal treatment protocol. Coupons were cut from the galvanized steel into 15 mm x 12 mm coupons, and the edges were bent to create a small well for holding the viral suspension. Metal coupons were then sterilized (via autoclave) and aseptically stored. In similar fashion, two-ply 100% cotton fabric was obtained and cut into 15 mm x 15 mm slices, sterilized (via autoclave), and aseptically stored.

Treatment using ClO2 gas was prepared according to ICA-Trinova (Newnan, GA) mixing protocol. Measured equal amounts of reagent A and B were mixed together in a gas permeable bag and hung on a clip inside the gassing container. Substrate of interest (either metal or cloth) was impregnated with MS2 bacteriophage and was placed on top of a test tube rack in the 5 L gassing chamber in which a small battery-operated fan was placed to promote equivalent gas distribution. In subsequent protocols, to ensure humidity stabilization, single-use humidity chips (Vetovation Raleigh, NC) were inserted into the testing chamber. Each treatment was performed in duplicate or triplicate along with untreated controls.

After treatment, the protocol for determining the number of bacteriophages recovered, killed, log-reduction, and percent reduction was based on the traditional viral plaque assay method (Figure 2). This method includes creating dilutions of the stock cultures, mixing the viral particles with the conjugate host cell, and using a double-layer agar technique. This method allows one to quickly quantify the number of viral particles per milliliter by counting the number of plaques present and using mathematical analysis to establish or determine the amount of virus recovered, killed, and inactivated in treated and untreated controls.

Treated and untreated substrates (containing bacteriophage) were placed in 4.5 mL of TSB and vortexed. The solutions were then diluted ten-fold 5 times in TSB.

Recoverable plaque forming units (PFU) were determined by delivery of 0.1 mL of the appropriate dilution into 4.0 mL of 1% nutrient soft agar. To this, 0.1 mL of *E. coli* (in logarithmic growth) was added to the soft agar/phage preparation and immediately poured onto the surface of TSA plates. Plates were incubated (37° C) overnight and recoverable PFU/mL were then determined from the treated and untreated substrate test coupons. A general outline of methodologies is outline in Figure 3.

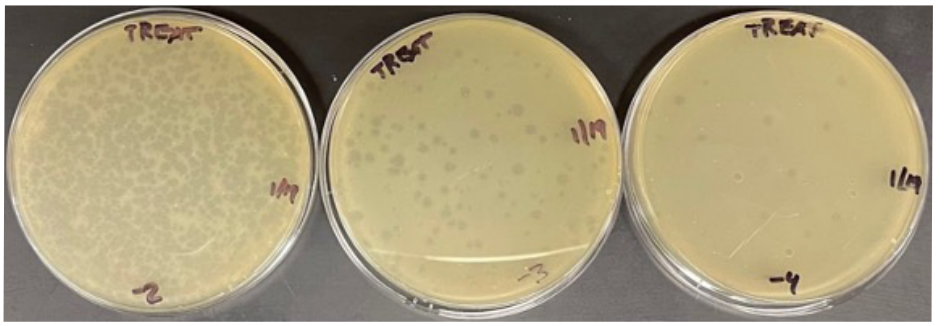

Figure 2: Example of plaque assay

Mathematical analysis calculations were performed to determine the amount of MS2 bacteriophage recoverable after treatment of a stock culture. For computations, plates with 30300 plaques were utilized. Plaques were tallied and documented in a laboratory-grade notebook. From the plaque assays, calculations were performed on the plate with the most countable plaques. The number of plaques counted was multiplied by ten (giving the number of recoverable viral particles per mL of that given dilution), then multiplied by ten, the number of times the number on the dilution tube (for example, if the number of plaques on a plate was 250: 250 x 10 = 2500 signifying PFU/mL in the -5 solution, the 2,500 is multiplied by ten, five times giving the number of recoverable viral particles in the stock solution and present from the material exposed to ClO2). Relative humidity determinations were made using a Thermo-Hygro meter (Fischer Scientific).

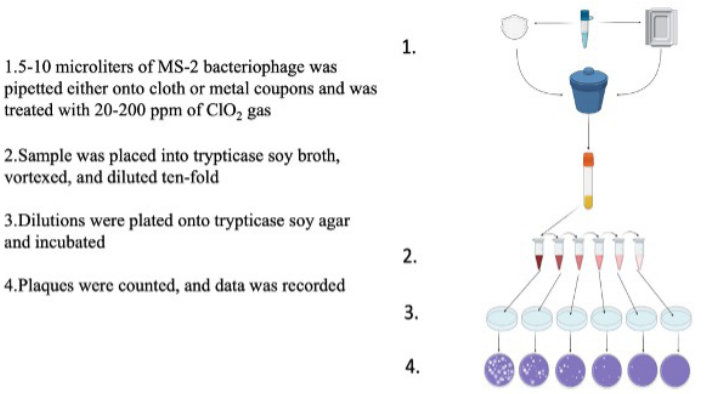

Figure 3: General outline of the methodologies developed and employed in the pursuit of the determination of the antiviral properties of ClO2 gas

Results

In the development of the ClO2 gas and MS2 bacteriophage testing protocol, preliminary studies were aimed at testing to evaluate if previous experimental protocols would be suitable for the proposed study. This included evaluation of studies published from the Environmental Protection Agency, Middle Tennessee State University, and other laboratories investigating the oxidative properties of ClO2. All tests were measured against controls that mirrored the protocol given to the test groups except for the exposure to ClO2 gas. These preliminary tests yielded results that promoted the collection of additional data in support of the antiviral potential of ClO2.

Studies were first aimed at the investigation of the antiviral properties of ClO2 on nonporous surfaces such as stainless-steel metal coupons. The investigation of metal coupons included the testing of 200 PPM ClO2 gas on small stainless-steel coupons yielding a percent reduction range of 61%-99.99% in recoverable MS2 bacteriophage at 200 PPM for either 45 minutes or 960 minutes (16 hours) gas treatment (Table 1). In this range, it was observed that allowing the MS2 bacteriophage suspension to dry to completion before gas treatment resulted in a decreased percent reduction with an average of 61% (*SD* = 0.093), while placing the coupon "wet" (not dried suspension) into the gassing chamber yielded a larger percent reduction with an average of 87% (*SD* = 0.024) at 45 minutes (Figure 4).

In optimization of these findings, it was observed that the organic content present in the MS2 bacteriophage suspension can alter the percent reduction of MS2 bacteriophage in the presence of ClO2 gas. For example, when the stock culture from

ATCC was diluted 1:10 in deionized water (thus reducing the organic content by 90%), the percent killed was increased. It was found that dilution of the stock MS2 bacteriophage culture by 10-fold yielded a 99.80% reduction of MS2 bacteriophage after 200 PPM exposure for 45 minutes (Figure 5) and a 99.99% reduction of MS2 bacteriophage after 960 minutes of exposure.

In review of ClO2 gas antiviral potential using stainless steel coupons, it was found that ClO2 was capable optimally of acting as an antiviral agent on stainless-steel coupons under certain conditions, such as reduction of organic content in the MS2 bacteriophage suspension and dependent upon the state of the suspension (either evaporated or wet) on the substrate. Log reduction displayed an average of 0.91 log reduction (< 90% reduction) in MS2 bacteriophage on stainless-steel surfaces when the contaminate was immediately exposed to ClO2 gas and a 0.41 log reduction in MS2 bacteriophage when the viral suspension was allowed to evaporate to dryness. Additionally, it was found that an average of 2.7 log reduction occurred when the viral suspension was diluted by 90% in water, thus reducing the organic content of the suspension (Figure 6). Thus, in continued optimization of the antiviral system, the protocol was adjusted to contain both 1:10 diluted stock MS2 bacteriophage suspension, and solutions were not allowed to dry to evaporation.

Prior sets of studies determined that maximum antiviral potential was achieved when the stock solution was diluted in water (thus, reducing organic content) and when the material was inoculated and not allowed to dry before exposure to ClO2 gas. Thus, the next phase of investigation examined the antiviral potential of ClO2 gas on cloth (a porous substrate) under the same optimized treatment protocol. In testing three variables, concentration, exposure time length, and relative humidity, it was observed that all three influence the ability of ClO2 to act as an antiviral agent (Table 2).

First, the effects of ClO2 on cloth for 45 minutes at 200 PPM yielded an average of 95% viral reduction (SD = 0.894) and a 99.8 % reduction at 960 minutes (Figure 6). It was then determined that decreasing the concentration of ClO2 gas to 40 PPM for 960 minutes altered the antiviral ability of ClO2 gas to 24% (SD = 0.021) (Figure 7). Lastly, it was determined that increasing the relative humidity to that of ≥ 90% and decreasing the concentration to 20 PPM increased the antiviral capabilities to a complete elimination of recoverable viral particles (Figure 8).

Table 1: Investigation of the effects of 200 ppm ClO2 gas on MS2 inoculated stainless-steel coupons at room temperature and ≤ 64% relative humidity

Contaminated Substrate	Exposure Time (min)	Percent Reduction †
Dry Steel Coupon*	45	61%
Wet Steel Coupon**	45	87%
Wet Steel Coupon**	960	99.30%
Diluted Wet Steel Coupon***	45	99.80%
Diluted Wet Steel Coupon***	960	99.99%

* Contaminate left on metal coupon for 1 hour ± 15 minutes to allow contaminated surface to dry to evaporation.

** After inoculation, surface was immediately exposed to ClO2 gas.

*** MS2 bacteriophage solution was diluted with a 1:10 sterile water solution.

† Average triplicate experiments

Table 2: Investigation of the effects of ClO2 gas on MS2 impregnated cloth at room temperature and ≤ 64%- ≥90 % relative humidity

Exposure Time	Concentration (PPM)	Relative Humidity (%)	Percent Reduction	Log Reduction
45	200	≤ 64%	95%	1.9
960	200	≤ 64%	99.8%	2.9
960	40	≤ 64%	24%	0.1
960	20	≥ 90%	NRV* (≈100%)	≥ 7

* NRV = No Recoverable Viruses

† Average duplicate experiments

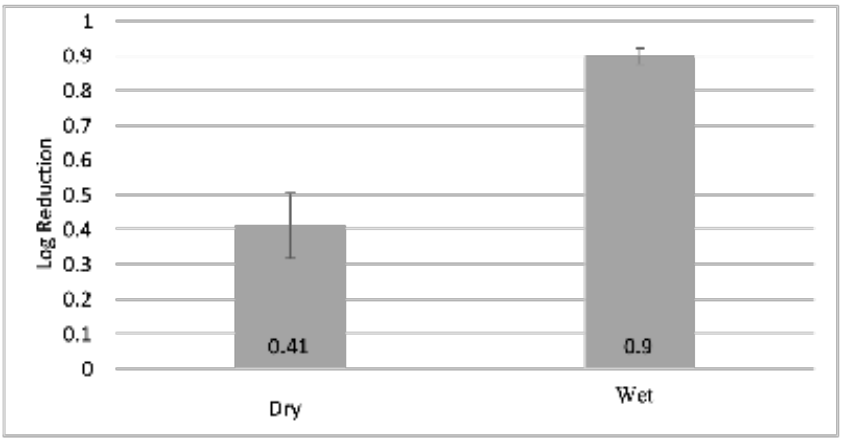

Figure 4: Examination of the effects of wet versus dry viral suspension contaminated stainless steel surfaces exposed to 200 ppm ClO2 at room temperature and ≤ 64% humidity for 45 minutes.

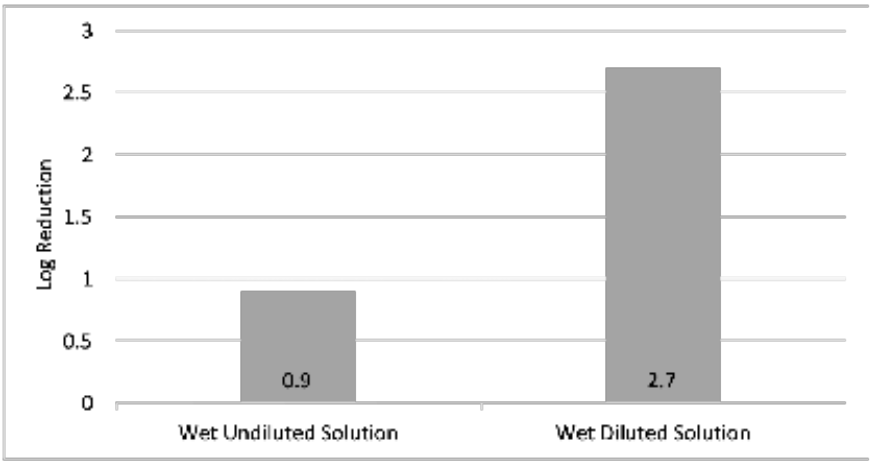

Figure 5: Examination of the effects of 1:10 diluted MS2 bacteriophage stock solution with sterile H2O prior to 200 ppm ClO2 gas exposure for 45 minutes at room temperature and <64% humidity

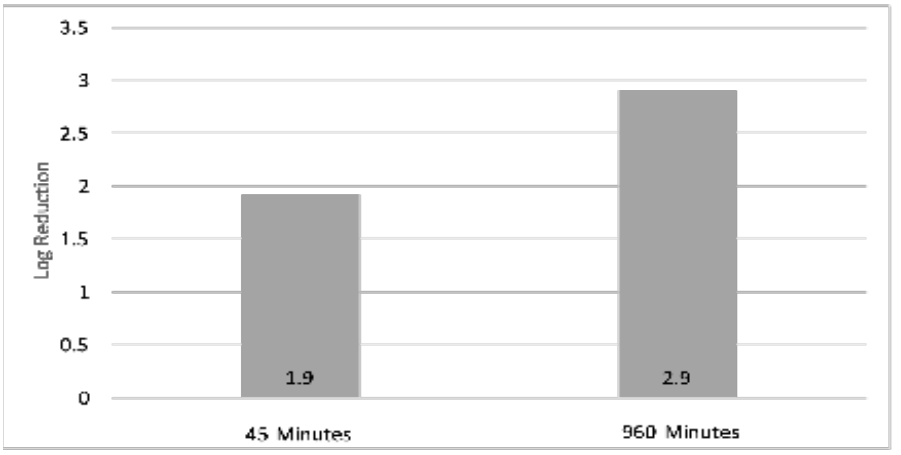

Figure 6: Examination of the antiviral capabilities of ClO2 on cloth impregnated with 1:10 diluted MS2 bacteriophage solution at 45 minutes and 960 minutes at room temperature and ≤ 64% relative humidity.

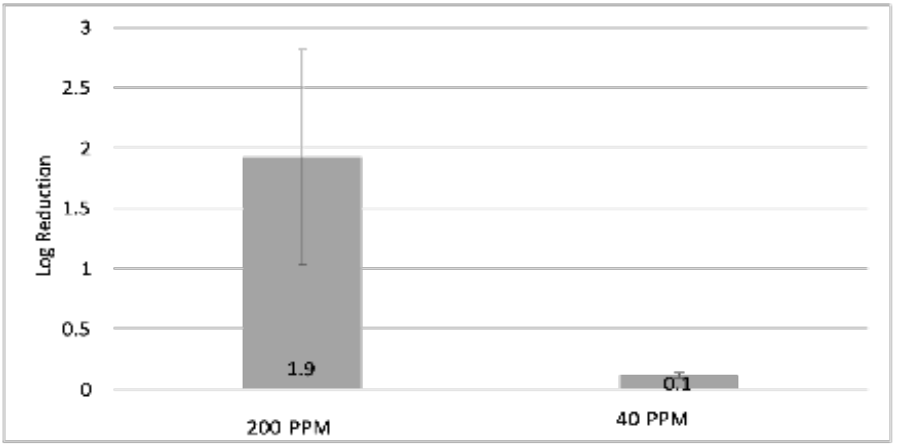

Figure 7: Examination of the antiviral capabilities of ClO2 on cloth impregnated with 1:10 diluted MS2 bacteriophage solution at varying concentrations of 200 PPM and 40 ppm ClO2 Gas for 45 minutes at room temperature and ≤ 64% relative humidity

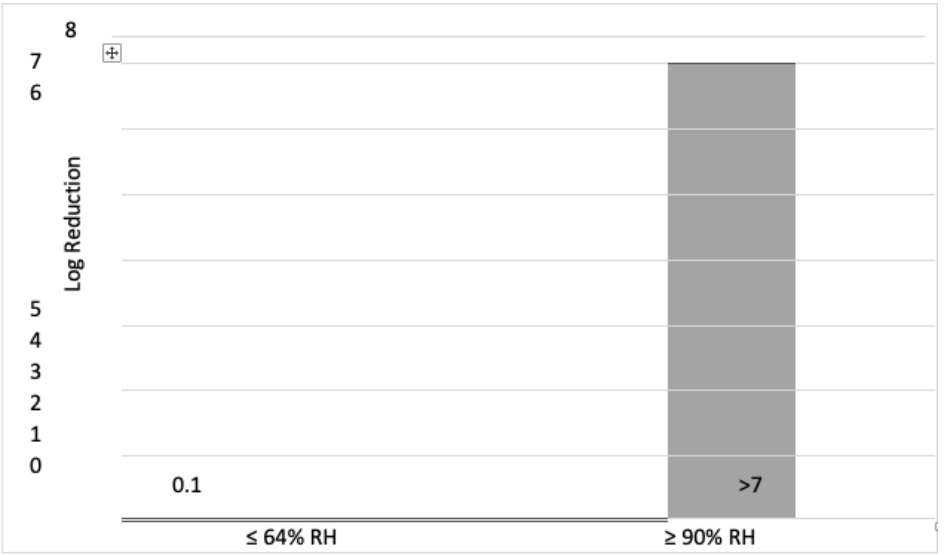

Figure 8: Examination of the antiviral capabilities of ClO2 on cloth impregnated with 90% diluted MS2 bacteriophage solution at varying relative humidity conditions of ≤ 64% and ≥ 90% relative humidity (RH)

Discussion

In summary, it was found that several factors influence the ability of ClO2 gas to act as an antiviral agent. Such factors that contribute to the gas's antiviral activity include the amount of organic content in which viral particles are suspended, humidity, concentration of ClO2 gas, and substrate. The optimal results of the antiviral capabilities of ClO2 gas were found at ≥ 90% relative humidity at room temperature with viral particles diluted in 90% sterile H2O at 20 PPM of ClO2 gas impregnated in cloth.

Initial findings revealed that percent reduction of viral particles is heavily dependent upon the organic concentration of the viral suspension. Since ClO2 gas is a strong oxidizing compound, especially toward organic matter, it would be expected that the ClO2 gas may be reduced to chlorine monoxide or to the chloride ion in the presence of higher levels of organic content (Hupperich et al., 2020; Wenk et al., 2013). The ability of ClO2 to oxidize organic matter implies that the viability of ClO2 gas to act as an antiviral agent is reduced in the presence of organic content; thus, as the results show, reduction of the organic content has a direct effect on the ability of ClO2 gas to inactivate the MS2 bacteriophage.

Additional findings revealed that ClO2 gas had better ability acting as an antiviral agent on cloth rather than stainless steel metal coupons. Conceivably, this may be

due to oxidation of the stainless steel by ClO2 gas, although additional research is needed to come to such conclusion. One other potential theory is that ClO2 gas penetrates deeper into the cloth thus being exposed to more viral particles leading to an increase in viral deactivation.

Lastly, it was found that ClO2 gas's ability to act as an antiviral agent is dependent upon the relative humidity of the environment in which it is deployed. This finding confirms other studies investigating ClO2 gas's ability to act as an oxidizing agent towards biological agents at varying relativity humidity (Park et al., 2018; Shirasaki et al., 2016). Studies suggest that this property of ClO2 gas is due to the varying solubilities of ClO2 gas at differing levels of humidity.

From the results, data supports the use of ClO2 gas as an antiviral agent. The results yielded a sufficient disinfection of both cloth and metal to be considered an antiviral agent. This study offers insight into the various factors that must be considered when using ClO2 gas as an antiviral agent and future obstacles that must be considered in pursuit of further ClO2 gas antiviral research.

Such application of the findings include use of ClO2 gas on metal and cloth as a potential antiviral agent but also suggest that there may be a wider horizon of antiviral application for ClO2 gas. Future studies should be aimed at investigation of ClO2 gas on surfaces such as wood and plastics. It also may be of interest to expand research on the use of ClO2 gas on bacteria' spores and mycobacteria which are known to be especially resistant to chemical inactivation.

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