The Effectiveness of Chlorine Dioxide in Inactivating Influenza Virus

by

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ABSTRACT

National outbreaks of avian influenza type A viruses have been the source of a wide range of adverse effects for the country. Not only do the deadly outbreaks lead to the culling of thousands of birds, economic damage to commercial farms, increased product prices, and blocked international trade, but there is also concern for human health, as some viruses have the ability to mutate and change from their usual host. The solution to the avian influenza outbreaks would be to find an efficient way to control and prevent pathogenic viruses from entering the chicken farms and causing disease. Currently, this solution does not exist on a large scale. Chicken or other poultry farms house thousands of birds in a small of area, so transmission is rapid and almost unavoidable once the virus is introduced. This study follows an approach using the chemical compound chlorine dioxide to treat and disable influenza viruses. Chlorine dioxide is known to have antibacterial and antiviral activity and is used as a disinfectant in a wide variety of settings, including military and medical. In order to test the effects chlorine dioxide has on influenza viruses, this study exposed seven different strains of influenza to chlorine dioxide gas. Among the strains used, two avian strains, two human strains, and three swine strains were tested. Each strain was exposed to different concentrations of chlorine dioxide, ranging from 50 parts per million to 500 parts per million, and exposed for different durations of time, ranging from 30 minutes to 4 hours. Each specific set of conditions was repeated three times. A hemagglutination assay was conducted after each trial to determine the viral titer. The effectiveness of each treatment depended on the length of exposure and the concentration. The treatments with a lower concentration

consistently inhibited the hemagglutinin protein completely when treated for four hours. As the concentrations increased, the length of exposure necessary to bring the virus titer to zero decreased. When completing trials with 500 parts per million, the assay showed complete hemagglutinin inhibition and therefore protection for influenza viruses in just thirty minutes of exposure. These results show that chlorine dioxide has high potential for a successful preventative treatment in poultry flocks. The granular form, which involves the chlorine dioxide compound and an oxidizing agent, is more advantageous than other methods of distributing chlorine dioxide treatment. This method of release for chlorine dioxide could be directly placed beneath the floor of poultry farm cages and be used as an ongoing method of prevention. These results show potential for the use of chlorine dioxide in this field.

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INTRODUCTION

Influenza A virus infects many different hosts, including humans, swine, birds and horses (Mehle, 2014). In humans, influenza A attacks the respiratory system, causing a wide variety of symptoms, including fever, headaches, and congestion. However, type A avian influenza results in a gastrointestinal illness for birds. Avian infection is the most widespread, and it has been reported that all strains of influenza A are derived from wild aquatic birds (Webster et al., 1992). Influenza is classified into 4 types, A, B, C and D. This project focuses on Type A, which is more severe and is the type of flu that comes to mind when we become infected. Type B influenza viruses are morphologically the same as influenza A viruses, but they only affect humans, and on rare occasions seals (Hinds et al., 2015). The important differences between A and B viruses are that type B is significantly milder, has low rates of antigenic drift, and does not cause epidemics (Hinds et al., 2015). Influenza C viruses are much less virulent than types A and B, and are associated with infections in children that are often asymptomatic (Principi et al., 2012). If symptoms do occur from these viruses, they appear in the upper-respiratory tract and are generally mild (Principi et al., 2012). Recently, an influenza type D virus was discovered in cattle in France, and it resembles the genome of type C viruses more closely than the other types (Ducatez et al., 2015). Type A viruses are characterized by the proteins on the capsid, or outer coat, of the virus. These subtype-determining proteins are Hemagglutinin (HA) and Neuraminidase (NA), which are also pivotal in the infection and spread of the virus (Figure 1). For example, a virus with HA 3 and NA 2 would be called H3N2. Of the 18 known HAs and 11 known NAs, 16 HAs and 9 NAs can be found

in birds (Mehle, 2014). Influenza can be further sub-categorized by the level of virulence. Most infections in birds are asymptomatic and are known as “low pathogenic avian influenza” (LPAI), while the “high pathogenic avian influenza” (HPAI) strains are associated with the outbreaks seen in the news, resulting in increased avian mortality (Capua and Alexander, 2006). The recent outbreaks in the United States have been due to HPAI viruses and characteristically have a 90-100% mortality rate in untreated poultry flocks (Webster and Govorkova, 2014).

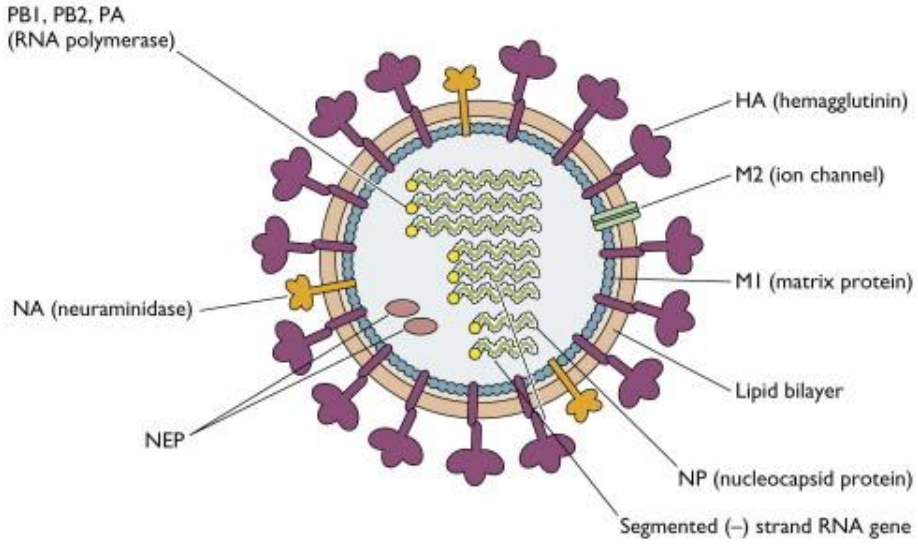

Figure 1: Basic structure of influenza virus, showing the hemagglutinin and neuraminidase surface proteins (Virology Blog 2009).

Regulation and eradication are ongoing processes and more research is needed to find an efficient and effective way to treat poultry flocks. Chlorine dioxide, which has been widely used in United States military as a bacterial disinfectant, is a preventative

measure that has been reported to inactivate influenza by inhibiting certain proteins (Doona et al., 2015). Researchers in Japan found that the antiviral activity of chlorine dioxide was nearly 10 times higher than sodium hypochlorite, another chemical compound frequently used for disinfecting and bleaching (Sanekata et al., 2010). Another study showed that chlorine dioxide was successful in inactivating various pathogens including enveloped viruses in the wet state, similar to the influenza viruses being tested in this study (Morino et al., 2011). The current task is to find an efficient way to distribute such a treatment to poultry flocks. Chlorine dioxide in aerosolized form has been found to be effective at inactivating the virus by interfering with the function of the hemagglutinin protein (Ogata 2012). The HA protein is key to infection of the host cells as it recognizes and binds to sialic acid on the host cell membrane (Figure 2) (Ogata and Takashi, 2008). Sialic acid exists at the end of carbohydrate chains on host cell membranes, making it the key part of the receptor (Ayora-Talavera et al., 2014). If the HA protein is inactivated, no sialic acid binding occurs, and no infection occurs. Our research uses chlorine dioxide in granular form for convenience purposes because aerosolized ClO_2 presents numerous challenges (Stubblefield and Newsome 2015). One of these challenges is the low stability of the gas, which requires preparation to be at the application site (Stubblefield and Newsome 2015). Previous research done with inactivation of influenza by chlorine dioxide has been very limited, but other uses for the chemical are being used. Chlorine dioxide was found to be highly successful in inactivating influenza A viruses, types H1N1 and H5N1, found in water supplies (Lénès et al., 2010), and has been approved by the Food and Drug Administration for water treatment since 1944 (Singh et al., 2002). Successful testing of chlorine dioxide against

pathogens causing food-borne illness, including *Escherichia coli* O157:H7, *Listeria monocytogenes, Shigella, Salmonella,* and hepatitis A virus, has encouraged wider use of the chemical in the food industry (Singh et al., 2002). In addition to being a more successful antibacterial and antiviral agent than current methods, chlorine dioxide does not form harmful byproducts or foul smells, and it reacts with organic matter at a faster rate (Singh et al., 2002). Chlorine dioxide exposures are regulated by the Occupational and Health Safety Administration, which limits human exposure for an 8-hour day to 0.28 milligrams per cubic meter (ATSDR, 2004). Levels of chlorine dioxide in treated drinking water are regulated by the Environmental Protection Agency, and the maximum level allowed is 1 milligram per liter (ATSDR, 2004). Both of these concentration limits are much higher than the levels used in our trials.

The influenza A virus genome contains eight segments of single-stranded RNA, which code for 10 gene products, all of which are necessary for successful infection (Webster et al., 1992). As illustrated in Figure 1 above, one of these genomic segments codes for a hemagglutinin protein that is expressed in the outer envelope of the virus. The binding site of hemagglutinin has the shape of a shallow groove and is made up of a 190-alpha helix, a 130 loop, and a 220 loop (Ogata, 2012). These numbers are based on approximate numbers of amino acid residues present. Another, and perhaps the most important, element of the binding site of hemagglutinin for our research purposes is a conserved amino acid residue called W153 (tryptophan 153), located at the bottom of the binding site (Ogata, 2012). There are many parts of the hemagglutinin receptor binding domain; collectively, this domain recognizes the specific sialic acid linkages on the host's membrane to prepare for viral entry (Yang et al., 2013). Efforts by Ogata found through

mass spectrometry that the W153 residue fragment was 32 mass units greater than expected after the virus was treated with chlorine dioxide. This change in mass units was due to the oxidation activity caused by chlorine dioxide treatments (Ogata, 2012). In almost every protein, form determines function, and when the shape of a protein is altered, its function is usually lost. That is the case with this hemagglutinin protein as well. This key part of the protein that binds to the sialic acid region on host cell changed conformations, and therefore is unable to make that essential initial linkage.

According to the Centers for Disease Control and Prevention, in December 2014 there were confirmed detections of pathogenic avian influenza in the United States, and by June 2015, 22 states had confirmed cases of a HPAI virus, many of these cases being found in poultry (CDCa). There is currently no efficient way to treat influenza on a large scale in poultry, leaving few options but to euthanize entire flocks of chickens or turkeys. In a recent case, a high-pathogenic H7N8 outbreak caused about 60,000 birds to be culled in an Indiana turkey farm (CIDRAP, 2016). The recent outbreaks have caused many foreign countries to ban poultry and egg products from the United States, and at home the prices of these products have risen drastically (CDCb). Another concern is human health. Avian influenza infections in humans are extremely rare, and, when they do occur, they have a low chance of being transmitted to another human. However, there is still concern as infection is possible when a human has contact with an infected animal. In order for an influenza virus to infect a cell, whether the host is human, avian or swine, the hemagglutinin protein must bind to sialic acid on the host cell membrane (Ogata and Takashi, 2008) (Figure 2). In humans, this binding occurs at the 2,6 linkage, but in birds, the linkage occurs at 2,3. Swine can have both of these linkages. Although rare, there is

chance for the combination of avian and human viruses to genetically mix in swine, and when this happens, a new, highly pathogenic virus may be formed (Mehle, 2014). For this to happen, the pig would have to be simultaneously infected with both an avian and human influenza virus (Webster et al., 1992). These conditions seem to be rare, and it is important to note that not all pandemic strains are a result of this genetic mixing. Nevertheless, swine are a common and susceptible host for human influenza viruses, and can act as a recombination vessel for new strains of virulent influenza viruses (Webster et al., 1992).

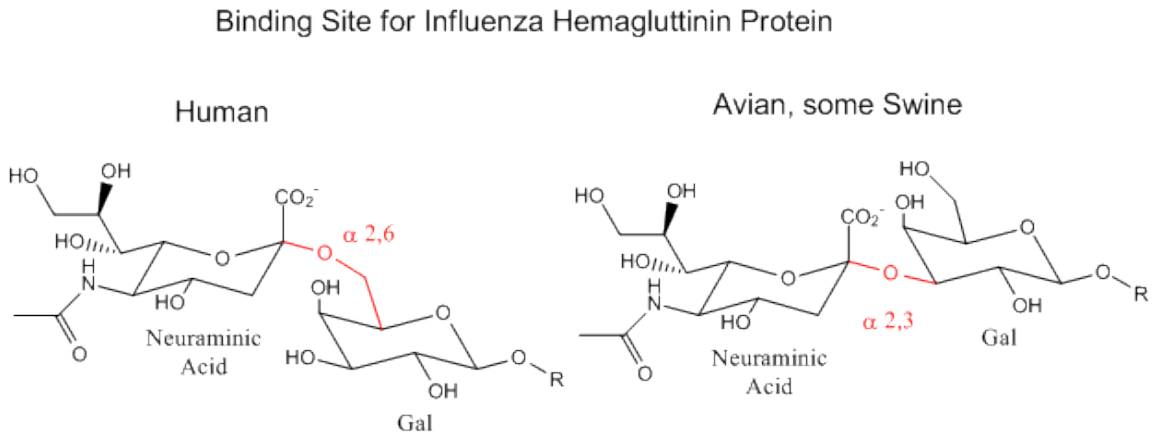

Figure 2: The difference between the sialic acid linkages in human and avian cells (UC Davis).

Examples of this genetic recombination in history include the Spanish Flu in 1918, killing an estimated 50 million people. The 1957 outbreak of H2N2 killed nearly 2 million people, and the 1968 strain of H3N2 was responsible for approximately 1 million

deaths (Pandemic Awareness). Each of these pandemics was due to an acquired gene segment and reassortment to create a new hemagglutinin and neuraminidase protein to which the human population had little to no immunity (Webster and Govorkova, 2014). In August of 2016, the World Health Organization reported that an additional five cases, with one death, of the avian influenza A strain H7N9 have been identified in addition to the total 798 since 2013. Another strain of concern is H5N1. To date, there have been 844 human infections with H5N1 and 499 deaths, a mortality rate of over 50% (WHO, 2015).

Some of the factors that cause the virus to be spread so rapidly are due to its own nature, like the ability to mutate to infect different hosts, while some are due to human interaction with the virus. One of the virulence factors that is characteristic of influenza viruses is its high rate of mutation. Subtypes are determined by proteins on the viral membrane, specifically with the proteins hemagglutinin and neuraminidase. Viral RNA polymerase is inclined to higher rates of error when polymerizing the RNA strands, causing hemagglutinin proteins to experience rates of mutation of around 2×10^{-3} base pair substitutions per position, per virus generation (Webster et al., 1992). Among the known different hemagglutinin proteins, the HA1 variable region is estimated to vary up to 30% (Webster et al., 1992). Evolutionarily speaking, the mutation of the hemagglutinin proteins is the virus' defense against host cell responses, since this protein is essential for infection and entry into the host, creating a coevolution of host and pathogen (Webster et al., 1992). Beyond the genetics of the virus, the physical transmission from host to host allows the virus to spread widely and quickly. Transmission of avian influenza viruses occurs through the feces of the bird, which can contain very high concentrations the viruses of up to 10^7 particles/gram and can hold infectious virus for more than 44 days

(Capua and Alexander, 2006). Within domestic poultry settings, humans play a large role in introducing and spreading viruses through the contact and movement of the caretakers, owners, staff, drivers, and delivery methods (Capua and Alexander, 2006). On a larger scale, disease has spread by wild bird migration and illegal international trading of poultry, eggs, and poultry products infected with HPAI viruses (Dong-Hun et al., 2016). Each of these factors contributes to the severity of all types of influenza strains, and calls for new methods of control and prevention to be studied and used in the near future.

While past experiments have used one virus, or limited variability in exposure time and concentration, our experiments evaluated multiple parameters. We used seven different host viruses, each tested at various concentrations of chlorine dioxide for several set lengths of time. The treatments, which are detailed in the procedure section, utilize gaseous form of chlorine dioxide and take place in a sealed environment. By taking into account all of these factors, we offer a more comprehensive study of chlorine dioxide and reveal more potential for its use in the future.

MATERIALS AND METHODS

Seven different strains of influenza virus were tested in this study for their infectivity following exposure to chlorine dioxide (Table 1). The naming process for influenza viruses is as follows: Type/Host*/Geographic Location/Isolate #/Year of Isolation

*not listed if human

As an example, A/Mal/NY/6750/78 describes a type A influenza virus isolated from a mallard in New York. This was isolate number 6750 in the year 1978. Genetic analysis is used to determine the host of origin, so the host of isolation and the determined host of origin may not match, as seen in Ty/IA/7352/80.

Virus	**Host of Origin**	**Subtype**
Mal/NY/6750/78	Avian	H2N2
Sw/Ont/2/81	Swine	H1N1
Sw/CO/1/77	Human	H3N2
Sw/CA/90	Swine	H1N1
Ty/IA/7352/80	Swine	H1N1
Ty/SD/7740/86	Avian	H1N1
Udorn/307/72	Human	H3N2

Table 1: Viruses Used in This Study

In order to conduct this study, viruses were prepared by growing them in embryonated chicken eggs. The following techniques were used to prepare and titer the viruses:

Candle the Eggs

Embryonated chicken eggs were obtained from a local farmer (Jim Rowland) in Murfreesboro, Tennessee. For successful propagation of the eggs, 9-11 day old fertile eggs were used. Each egg was held up to a candle box (Lyon Electric Company, Chula Vista, CA), allowing the air sac to be seen. This area was marked with a Sharpie to ensure the proper infection site.

Infecting the Eggs

The eggs were first sprayed with 70% ethanol (AAPER Alcohol and Chemical Co. Shelbyville, KY). to prevent bacterial contamination. Using a Dremel grinding tool, a hole was drilled just above the air sac and 0.1 mL of a virus stock was injected into the egg using a tuberculin syringe (Becton Dickinson, Franklin Lakes, NJ). The virus must be injected into the allantoic fluid for proper growth. Once the virus had been injected, the hole was sealed with melted paraffin (Paraplast Monoject Scientific, St. Louis, MO) and the eggs were returned to the incubator (G.Q.F. Manufacturing Company, Savannah, GA) for two additional days of incubation at 38°C. Following incubation, the eggs were placed in a refrigerator at 4°C for one day.

Harvesting the Eggs

The eggs were sprayed with 70% ethanol for disinfection. Forceps were used to crack and remove the shell above and around the air sac. The virus had been growing in the allantoic fluid, so the membranes and embryo were gently pushed to the side while a

pipette was used to extract the fluid. The allantoic fluid is placed into 15 mL centrifuge tubes (Becton Dickinson) and stored at 4°C until used for the hemagglutination assay.

Hemagglutination Assay

Serial 2-fold dilutions of allantoic fluid were made in phosphate buffered saline (PBS) using a 96 well tissue culture plate (Corning Assay Plate, Corning Inc., Corning, NY). PBS is prepared with the reagents outlined in Table 2, which were all obtained from Fisher Scientific, Fair Lawn, NJ.

NaCl	4.0 g
KCl	0.1 g
Na_2HPO_4	0.57 g
KH_2PO_4	0.1 g
dH_2O	495 mL

Table 2: Phosphate buffered saline composition

First, 100 µL of allantoic fluid was placed into the first column and 50 µL of PBS was placed into columns 2-12. Then, 50 µL of allantoic fluid from column 1 was pipetted and mixed into column 2, then 50 µL was taken from column 2 and mixed into column 3. This process continued until column 12, and the last 50 µL was discarded. Chicken blood was prepared from packed, washed red blood cells (RBCs) (Lampire Biological Laboratories, Pipersville, PA) and PBS to a concentration of 0.5%, and 50 µL was added to each well. After 40 minutes, the plates were analyzed. RBCs allowed quantification by microtiter of the viruses. The sialic acid receptors on the RBCs bound to the hemagglutinin protein found on influenza viruses and formed a lattice structure. When the red blood cells have stayed in suspension, that indicated the presence of flu virus

because hemagglutinin has the ability to agglutinate. If the plates showed a button of red blood cells, that indicated the absence of the virus (Figure 3). The dilution scheme also determines the concentration of virus (Figure 4).

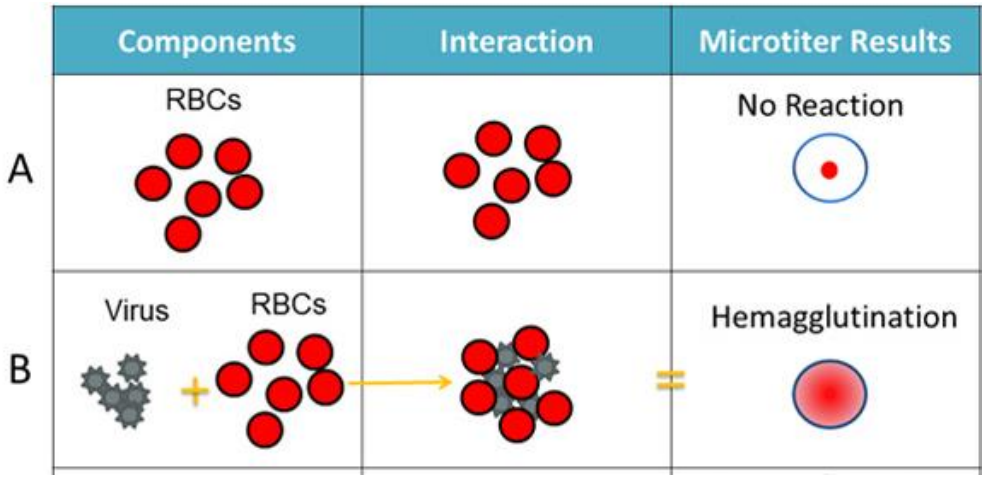

Figure 3: Viral interaction with red blood cells (CDC 2014).

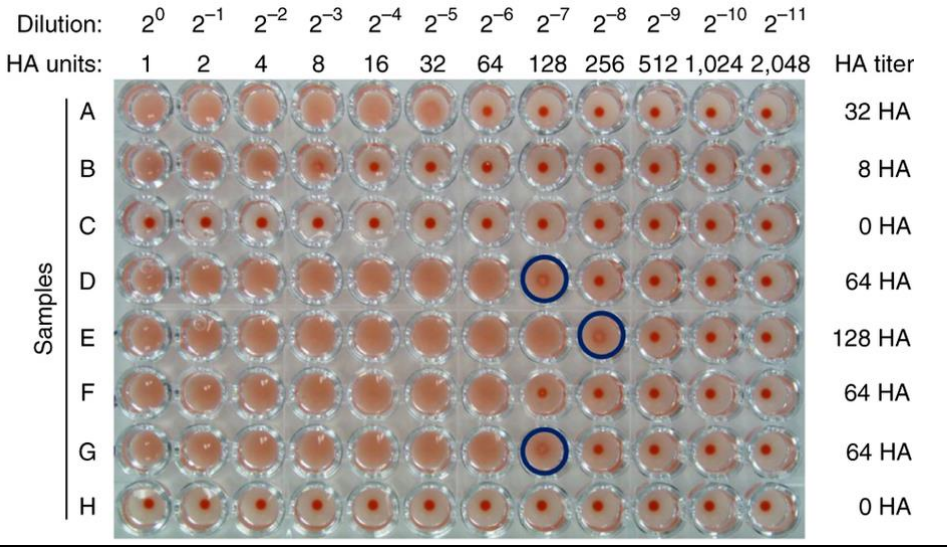

Figure 4: Hemagglutination Assay Example of Results (NPG 2014).

Chlorine Dioxide Evaluation

Three variables were tested:

1. Virus and subtype (Table 1)
2. Concentration of chlorine dioxide ranging from 50-500 ppm
3. Length of exposure time ranging from 30 minutes to 4 hours

Concentration of chlorine dioxide

Chlorine dioxide was used at concentrations ranging from 50 parts per million (ppm) to 500 ppm (50, 100, 500ppm). The concentration was manipulated by weight of the components, A and B, which were mixed together just before use in a fixed volume container (15 liters) (Figure 5).

Figure 5: Image of apparatus used to conduct trials.

The A and B components are provided by the company ICA TriNova (Atlanta, GA). Part A contains the chlorine dioxide in granular form, while part B is the oxidizing agent that lowers the pH and causes the chlorine dioxide to be released from part A. These concentrations were determined by consultation with the chlorine dioxide manufacturer (Joel Tenney, ICA TriNova, personal communication, February 2, 2016) (Figure 6). Levels of chlorine dioxide used are sub-toxic and therefore not harmful to humans.

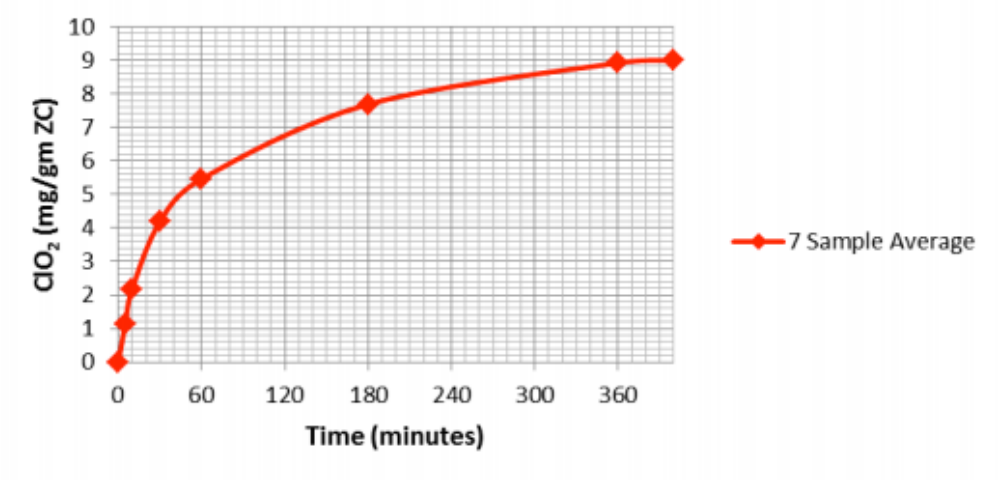

Figure 6: Average Release Rate of Chlorine Dioxide (mg$Cl0_2$/gm of precursor).

Time of Exposure

The time of exposure of the virus ranged from 30 minutes to 4 hours (30 minutes, 1 hour, 2 hours, 4 hours). These times were chosen based on consultation with the ClO_2 manufacturer.

Test Set-Up

Allantoic fluid (1.0 mL) containing each virus being tested was put into an open 35 mm x 10 mm petri dish (Becton Dickinson). The viruses are diluted in PBS to a standard HA titer of 16, approximately 16 million viruses per milliliter. Each petri plate was placed into the 15.4 L bucket along with chlorine dioxide at 50 ppm. After 30 minutes of exposure, the concentration of the virus was determined by hemagglutination assay. This was repeated at each concentration being evaluated. Similar testing was carried out with the time of exposure being varied. The goal was to determine the lowest concentration that completely inactivated the virus in the shortest time. A hemagglutination titer of zero indicates virus inactivation for the purposes of this study. Each treatment was repeated three times. A summary of different times and concentrations is in Table 2. A diagram of the experimental set up is shown in Figure 7.

Treatments	30 Minutes	1 hour	2 hours	4 hours
50 ppm	30min/50ppm	1 hour/50 ppm	2hours/50ppm	4hours/50ppm
100 ppm	30min/100ppm	1hour/100ppm	2hours/100ppm	*ND
500 ppm	30min/500ppm	1hour/ 500 ppm	2hours/500 ppm	*ND

*ND = Not Determined

Table 3: Treatments to be completed.

Percent Reduction

The percent reduction was calculated by averaging the titer results from each trial. That number was then subtracted from the original titer of 16. This was divided by 16, then multiplied by 100 to find the percent reduction of each set of trials. An example of this calculation is shown for Mallard, 50 ppm for 30 minutes:

Experimental = (trial 1 + trial 2 + trial 3)/3	(0+16+8)/3 = 8
(control – experimental) = x	(16-8) = 8
x/control titer = y	8/16 = 0.5
(y) (100) = percent reduction	(0.5) (100) = 50% reduction

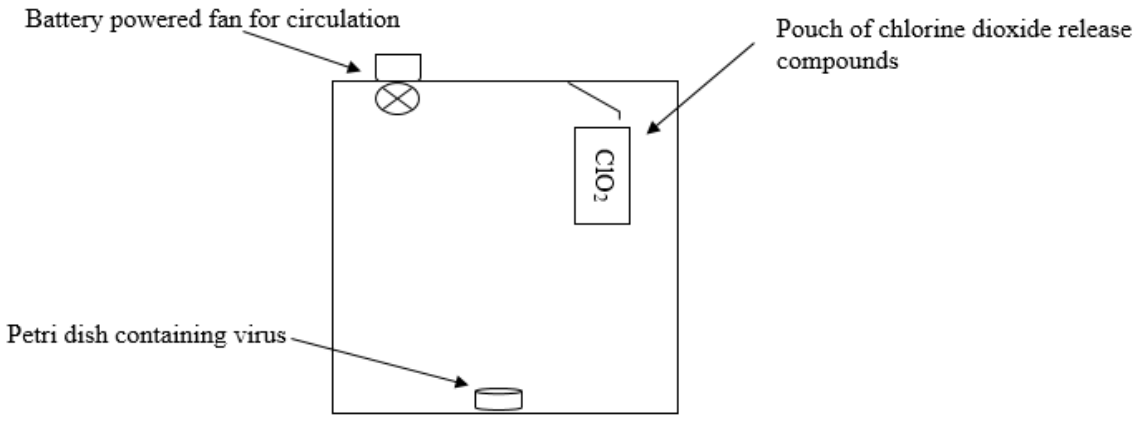

Figure 7: Diagram of experiential trial set-up.

RESULTS

Two avian virus strains were tested: Mal/NY/6750/78 and Ty/SD/7740/86.

Avian virus Mal showed a steady increase in percent reduction at 30 minutes, as concentration of chlorine dioxide increased (Figure 8). At the lowest concentration (50 ppm) and the lowest time of exposure (30 minutes) were applied to Mal, the percent reduction was 50%. When the same virus was tested at 500 ppm for 30 minutes, the percent reduction increased to 100%. When the virus was exposed to chlorine dioxide for 1 hour, the virus titer concentrations were completely reduced at every concentration, including 50 ppm (Figure 9). When Mal was exposed for 2 hours at 500 ppm, there was a slightly lower percent reduction (95.8%) than previous results, but the other concentrations showed 100% reduction (Figure 10). At a 4-hour exposure at 50 ppm, the Mal virus was reduced by 100% (Figure 11).

Avian virus Ty/SD had a very low percent reduction of only 16.7% when tested at 50 ppm for 30 minutes (Figure 12). This value was very low compared to the other avian virus tested, which was reduced by 50% under the same conditions. At 100 ppm for 30 minutes, the virus was still not completely reduced, and had a percent reduction of 58.3%. The treatment with 500 parts per million for 30 minutes, however, showed a 100% reduction of the virus. This virus showed some resistance to the treatment of 50 ppm for 1 hour with 66.7% reduction, but that increased to 100% reduction for both 100 ppm and 500 ppm (Figure 13). The results at each concentration for a 2-hour exposure were the same as the Mal virus, showing a slightly lower reduction at 50 ppm, and a

complete reduction at 100 ppm and 500 ppm (Figure 14). Also, similar to the avian virus Mal, when exposed to 50 ppm for 4 hours, the virus was reduced by 100% (Figure 15).

The two human virus strains that were tested were Sw/CO/1/77 and Udorn/307/72

When the human strain Sw/CO was tested at a 30-minute exposure to chlorine dioxide, the percent reduction increased as the concentration of chlorine dioxide was increased (Figure 16). At 50 ppm, the reduction was 79.16%, but for 100 ppm and 500 ppm, the reduction increased to 100%. Sw/CO was completely reduced at each concentration when exposed to chlorine dioxide for 1 hour (Figure 17). This virus was also completely reduced at each concentration when exposed to the chemical for 2 hours (Figure 18). However, when Sw/CO was exposed to the chlorine dioxide concentration of 50 ppm for 4 hours, there was a 95.8% reduction (Figure 19). Every other virus showed 100% reduction at this treatment.

The other human strain tested was Udorn. When Udorn was exposed for 30 minutes to 50 ppm of chlorine dioxide, the reduction was 50% (Figure 20). When the concentration was increased to 100 ppm, the percent reduction increased to 87.5%, and when the concentration was increased to 500 ppm, 100% reduction was achieved (Figure 21). Similar results were found when the same concentrations were tested for 1 hour. At 50 ppm, there was a 50% reduction, at 100 ppm there was an 83.3% reduction, and at 500 ppm, there was a 100% reduction of the virus (Figure 21). When the virus was tested at 50 ppm for 30 minutes, the reduction was found to be 66.7%, but this increased to 100% reduction for both 100 ppm and 500 ppm at the same length of exposure (Figure 22). Like most other strains, when the virus was exposed to 50 ppm for 4 hours, 100% reduction was achieved (Figure 23).

Finally, three strains of swine viral strains were tested: Sw/Ont/2/81, Sw/CA/90, and Ty/IA7352/80.

Sw/Ont was very susceptible to the chlorine dioxide treatments. At 50 ppm at a length of 30 minutes, the percent reduction was 91.7% (Figure 24). When the concentrations were increased to 100 ppm and 500 ppm, the virus was reduced by 100% (Figure 24). When the virus was tested for 1 hour at each concentration, the percent reduction was 100% for each concentration (Figure 25). Similarly, when Sw/Ont was exposed to chlorine dioxide at the set concentrations for 2 hours each, there was a 100% reduction for all three (Figure 26). Finally, when the virus was exposed to the treatment of 50 ppm for 4 hours, there was complete reduction (Figure 27).

Another swine virus that was tested was Sw/CA. During the 30 minute exposures at concentrations of 50 ppm, 100 ppm, and 500 ppm, the percent reductions achieved were 83.3%, 100%, and 100%, respectively (Figure 28). The next set of trials that exposed the virus to concentrations of chlorine dioxide for 1 hour showed that there was only a 62.5% reduction at 50 ppm (Figure 29). When exposed to chlorine dioxide at 100 ppm at 1 hour, the reduction increased to 95.8%, and when the concentration was increased to 500 ppm, the reduction increased again to 100% (Figure 29). When the trials were conducted at 2 hour lengths of exposure, the reduction was 95.8% at 50 ppm, but 100% reduction at 100 ppm and 500 ppm (Figure 30). When Sw/CA was exposed to 50 ppm of chlorine dioxide for 4 hours, 100% reduction was achieved (Figure 31).

The final strain of swine virus tested was Ty/IA. Similar to Sw/Ont, the only treatment that did not reach 100% reduction was at 50 ppm for 30 minutes (Figure 32). There was 100% reduction for each concentration when tested for 1 hour (Figure 33), as

well as 100% reduction for each concentration when tested for 2 hours (Figure 34). Finally, when the virus was exposed to 50 ppm of chlorine dioxide for 4 hours, 100% reduction was achieved (Figure 35).

In summary, in order for a 50 ppm concentration to be effective, it would have to be at the 4-hour time for most viruses. Any less than this, there was not enough consistency to determine that it would be effective enough. For a 100 ppm concentration, the minimum effective length of exposure was two hours. At this time, there was ability for viral infection. When the viruses were tested at 500 ppm, the treatment was completely effective even after 30 minutes.

		30 min		
		Trial 1	Trial 2	Trial 3
50 ppm	Mal	0	16	8
	Sw/Ont	0	0	4
	Ty/IA	8	4	2
	Sw/CO	0	2	8
	Sw/CA	4	4	0
	Ty/SD	8	16	16
	Udorn	8	8	8
100 ppm	Mal	2	2	2
	Sw/Ont	0	0	0
	Ty/IA	0	0	0
	Sw/CO	0	0	0
	Sw/CA	0	0	0
	Ty/SD	8	8	4
	Udorn	4	2	2
500 ppm	Mal	0	0	0
	Sw/Ont	0	0	0
	Ty/IA	0	0	0
	Sw/CO	0	0	0
	Sw/CA	0	0	0
	Ty/SD	0	0	0
	Udorn	0	0	0

Table 4: Viral titer results for each virus exposed to chlorine dioxide for 30 minutes at concentrations of 50 ppm, 100 ppm, and 500 ppm.

		1 hour		
		Trial 1	Trial 2	Trial 3
50 ppm	Mal	0	0	0
	Sw/Ont	0	0	0
	Ty/IA	0	0	0
	Sw/CO	0	0	0
	Sw/CA	2	0	4
	Ty/SD	4	8	4
	Udorn	8	4	4
100 ppm	Mal	0	0	0
	Sw/Ont	0	0	0
	Ty/IA	0	0	0
	Sw/CO	0	0	0
	Sw/CA	0	0	2
	Ty/SD	2	0	0
	Udorn	0	0	0
500 ppm	Mal	0	0	0
	Sw/Ont	0	0	0
	Ty/IA	0	0	0
	Sw/CO	0	0	0
	Sw/CA	0	0	0
	Ty/SD	0	0	0
	Udorn	0	0	0

Table 5: Viral titer results for each virus exposed to chlorine dioxide for 1 hour minutes at concentrations of 50 ppm, 100 ppm, and 500 ppm.

		2 hours		
		Trial 1	Trial 2	Trial 3
50 ppm	Mal	2	0	0
	Sw/Ont	0	0	0
	Ty/IA	0	0	0
	Sw/CO	0	0	0
	Sw/CA	0	2	0
	Ty/SD	0	2	4
	Udorn	0	0	0
100 ppm	Mal	0	0	0
	Sw/Ont	0	0	0
	Ty/IA	0	0	0
	Sw/CO	0	0	0
	Sw/CA	0	0	0
	Ty/SD	0	0	2
	Udorn	0	0	0
500 ppm	Mal	0	0	0
	Sw/Ont	0	0	0
	Ty/IA	0	0	0
	Sw/CO	0	0	0
	Sw/CA	0	0	0
	Ty/SD	0	0	0
	Udorn	0	0	0

Table 6: Viral titer results for each virus exposed to chlorine dioxide for 2 hours at concentrations of 50 ppm, 100 ppm, and 500 ppm.

		4 hours		
		Trial 1	Trial 2	Trial 3
50 ppm	Mal	0	0	0
	Sw/Ont	0	0	0
	Ty/IA	0	0	0
	Sw/CO	0	2	0
	Sw/CA	0	0	0
	Ty/SD	0	0	0
	Udorn	0	0	0

Table 7: Viral titer results for each virus exposed to chlorine dioxide for 4 hours at concentration of 50 ppm.

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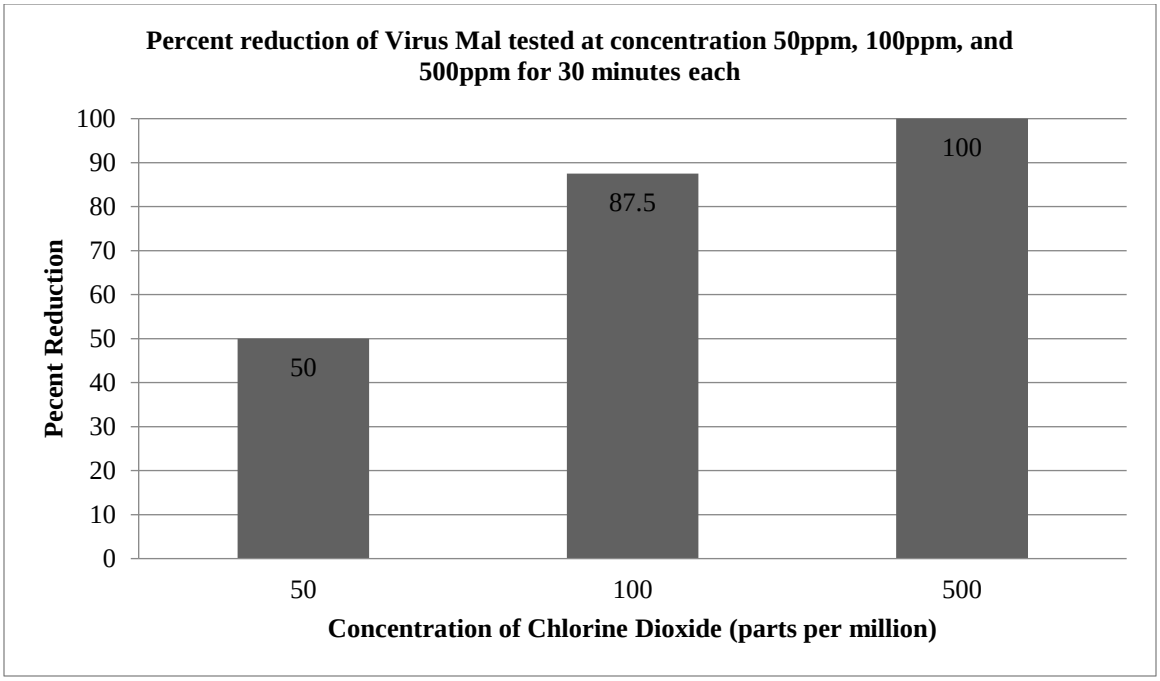

Figure 8: Calculated percent reduction of avian virus Mal/NY/6750/78 tested at concentrations 50 ppm, 100 ppm, and 500 ppm for 30 minutes each.

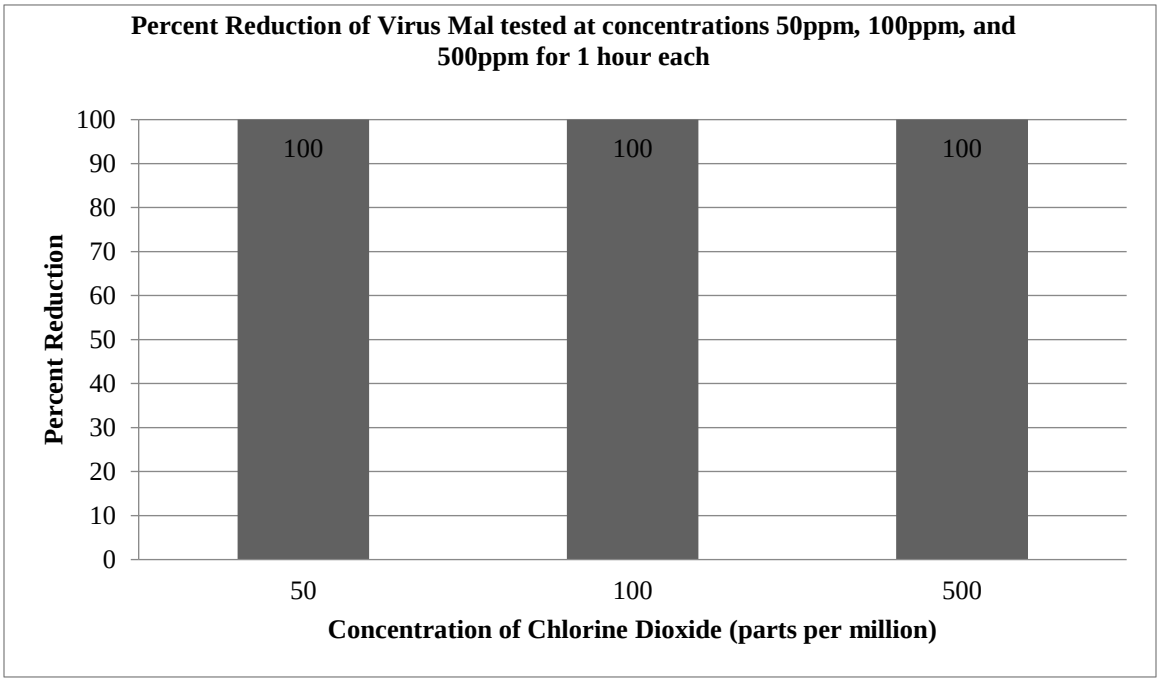

Figure 9: Calculated percent reduction of avian virus Mal/NY/6750/78 tested at concentrations 50 ppm, 100 ppm, and 500 ppm for 1 hour each.

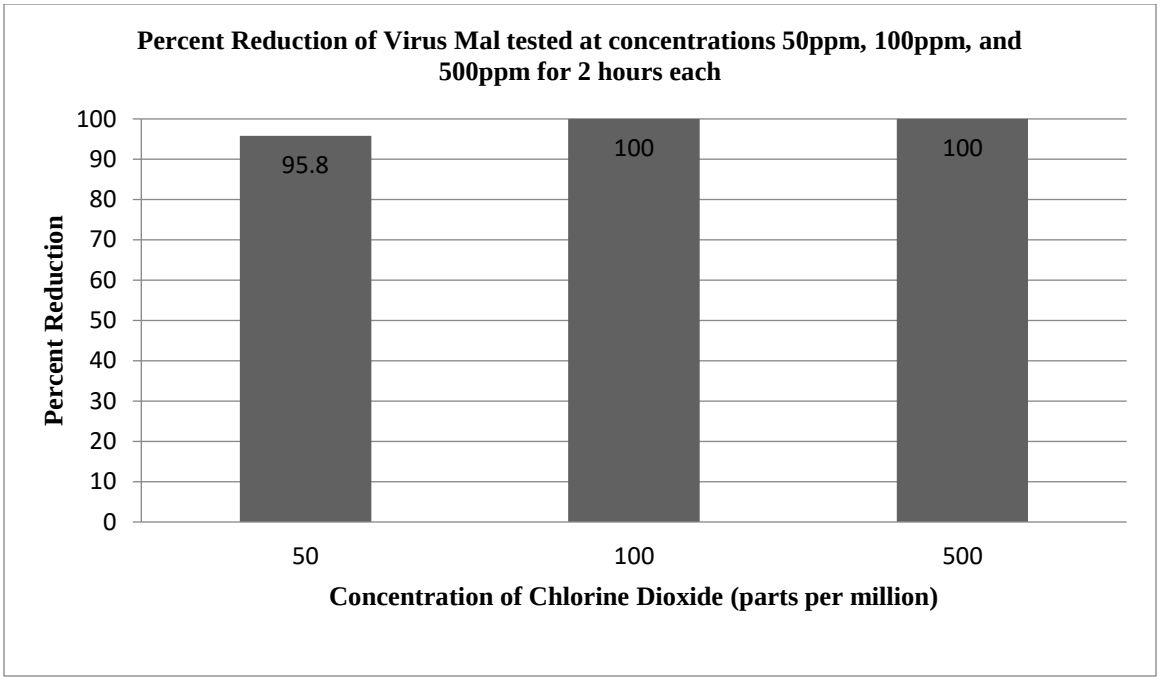

Figure 10: Calculated percent reduction of avian virus Mal/NY/6750/78 tested at concentrations 50 ppm, 100 ppm, and 500 ppm for 2 hours each.

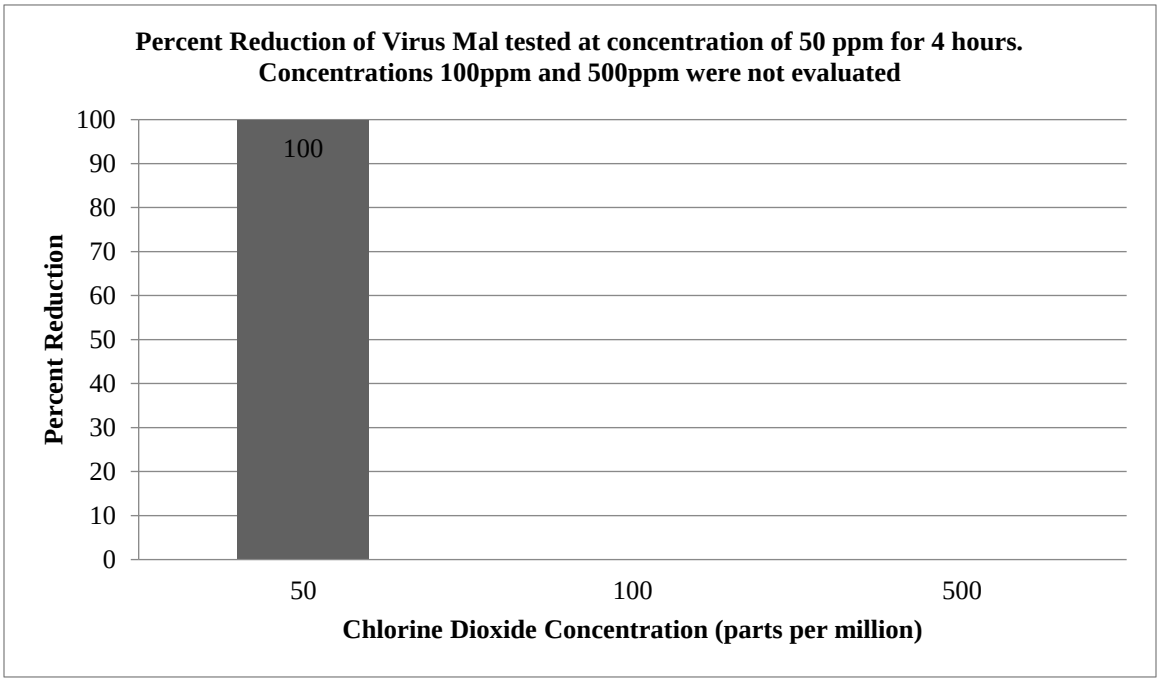

Figure 11: Calculated percent reduction of avian virus Mal/NY/6750/78 tested at concentration on 50 ppm for 4 hours. Concentrations of 100 ppm and 500 ppm were not evaluated at this time length because 100% reduction was achieved at 50 ppm.

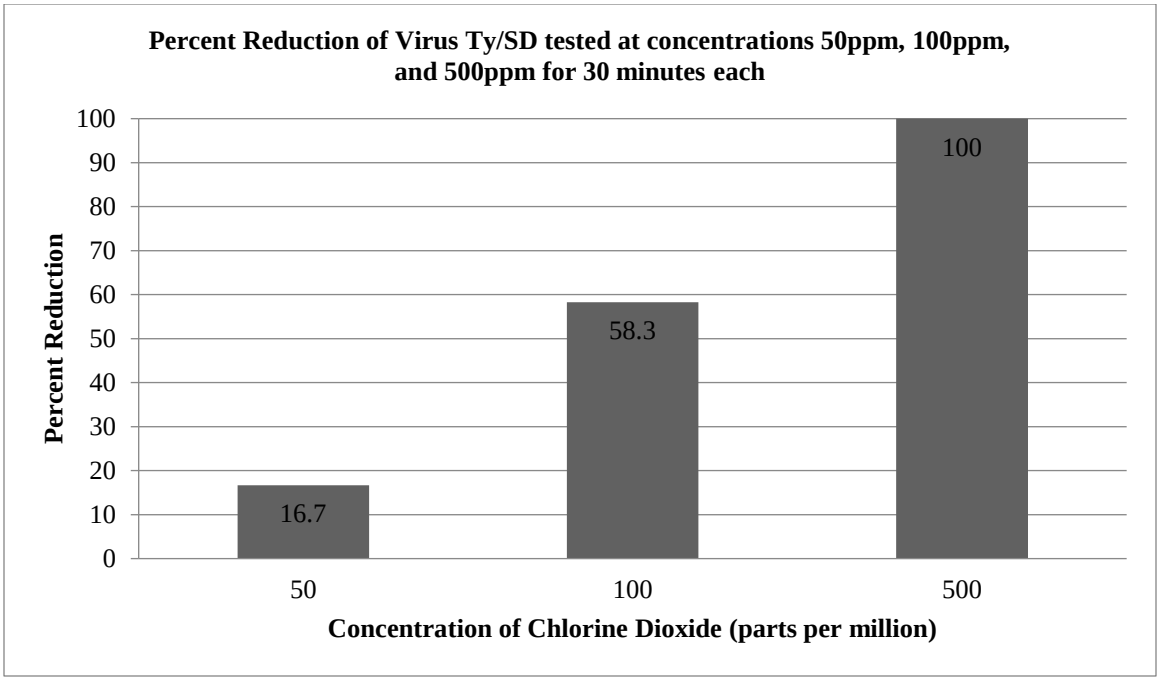

Figure 12: Calculated percent reduction of avian virus Ty/SD/7740/86 tested at concentrations 50 ppm, 100 ppm, and 500 ppm for 30 minutes each.

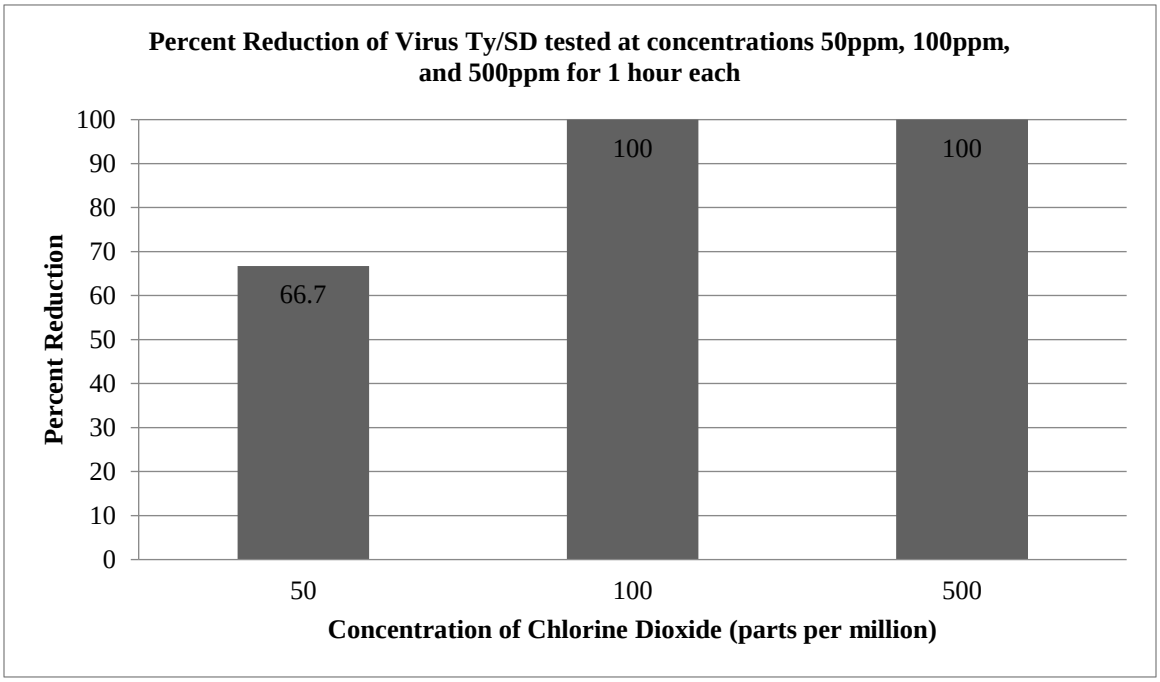

Figure 13: Calculated percent reduction of avian virus Ty/SD/7740/86 tested at concentrations 50 ppm, 100 ppm, and 500 ppm 1 hour each.

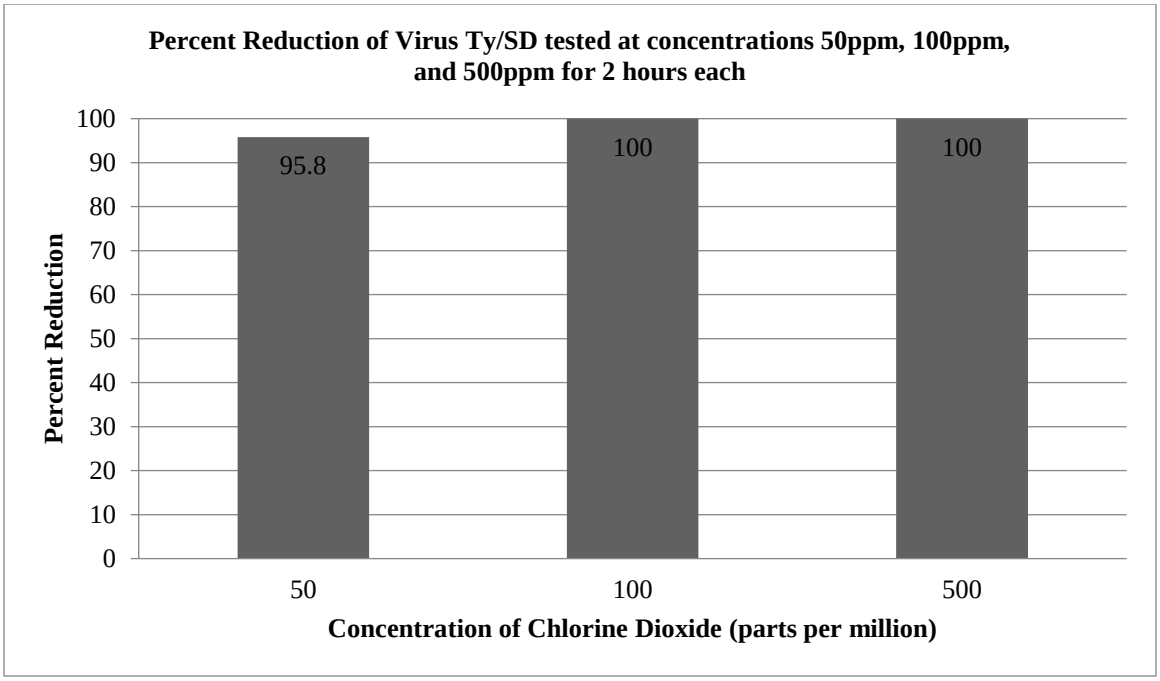

Figure 14: Calculated percent reduction of avian virus Ty/SD/7740/86 tested at concentrations 50 ppm, 100 ppm, and 500 ppm 2 hours each.

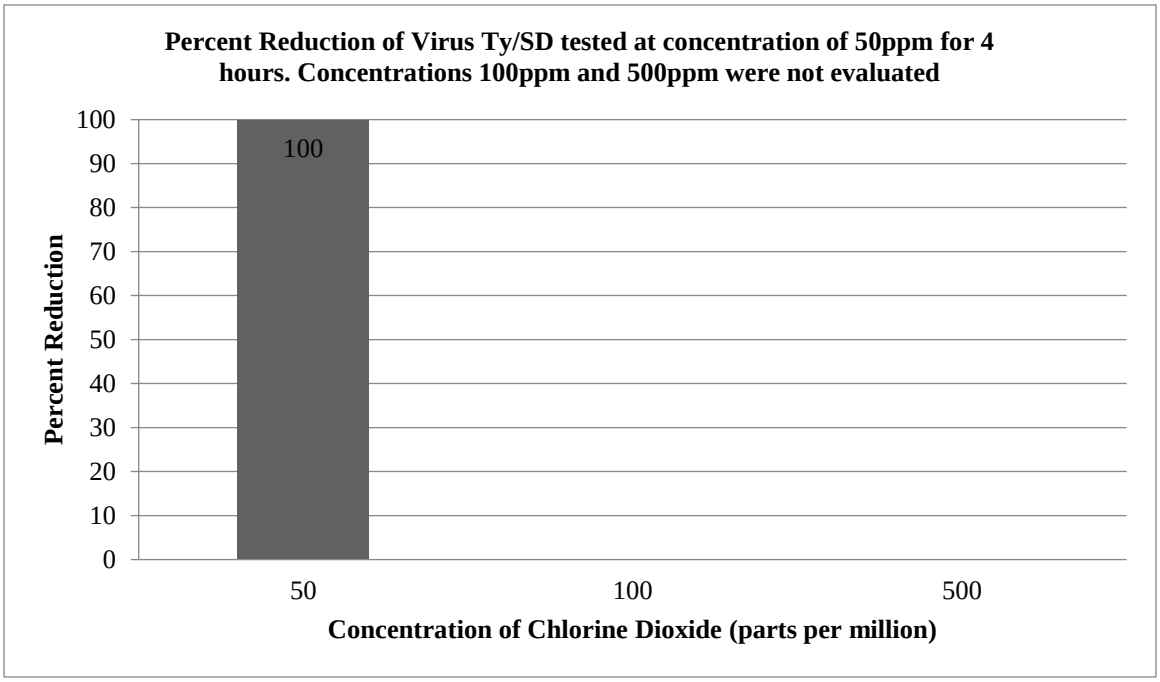

Figure 15: Calculated percent reduction of avian virus Ty/SD/7740/86 tested at concentrations 50 ppm for 4 hours. Concentrations of 100 ppm and 500 ppm were not evaluated at this time length because 100% reduction was achieved at 50 ppm.

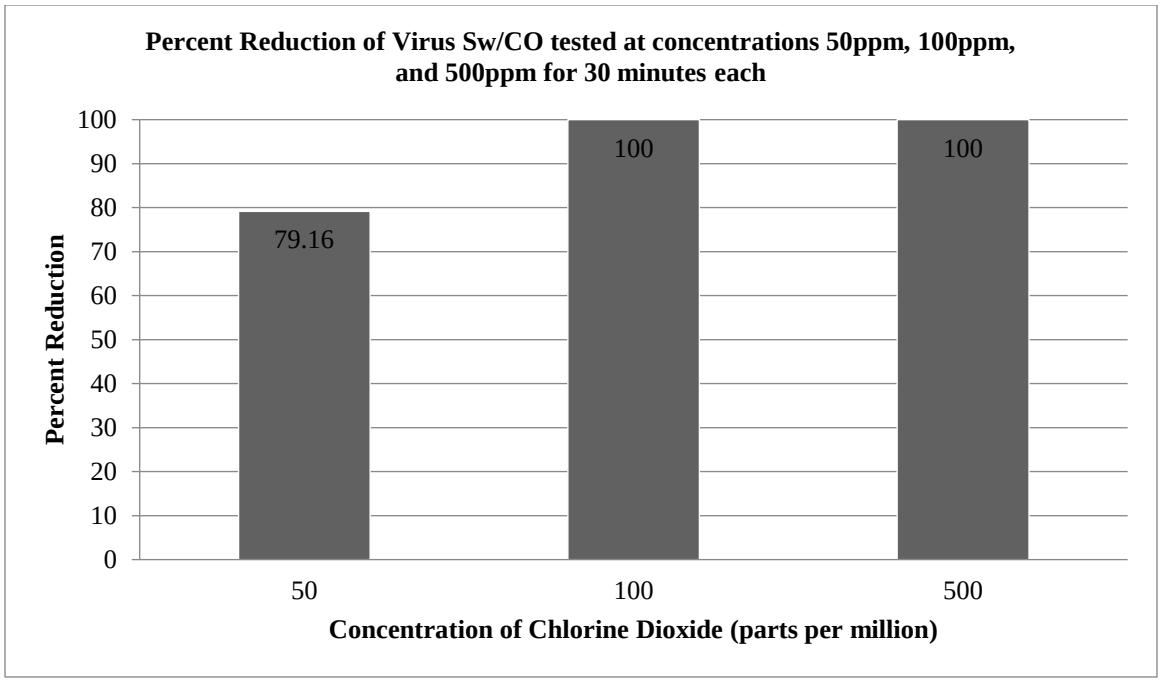

Figure 16: Calculated percent reduction of human virus Sw/CO/1/77 *tested at concentrations 50 ppm, 100 ppm, and 500 ppm for 30 minutes each.*

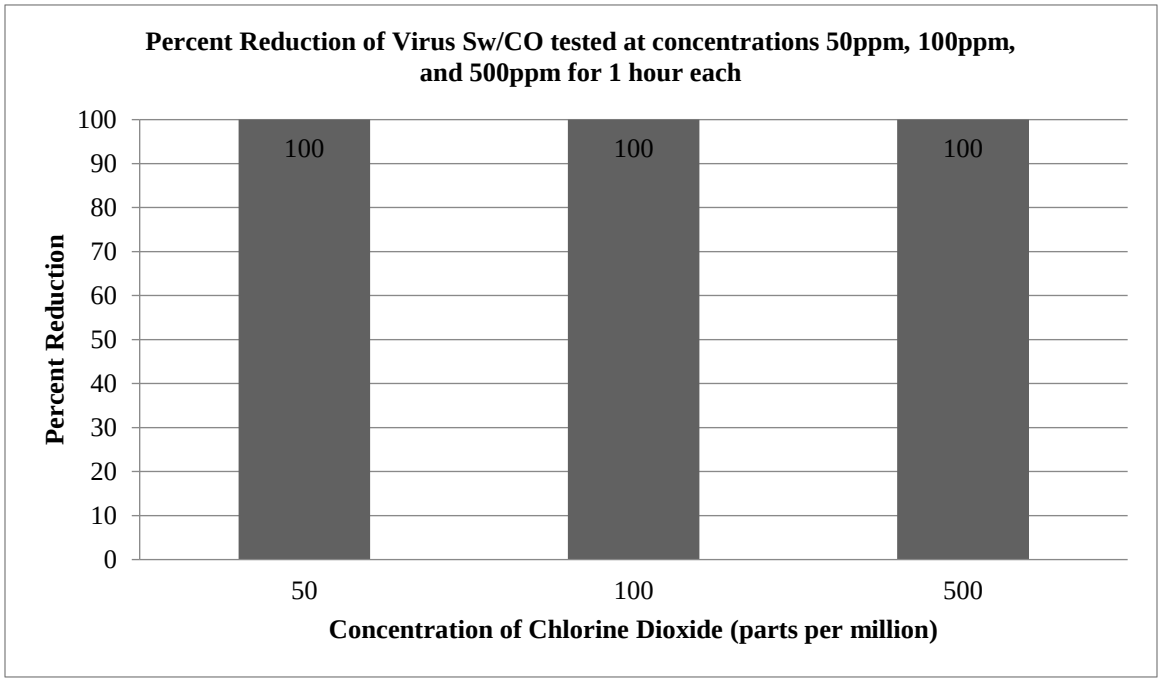

Figure 17: Calculated percent reduction of human virus Sw/CO/1/77 *tested at concentrations 50 ppm, 100 ppm, and 500 ppm for 1 hour each.*

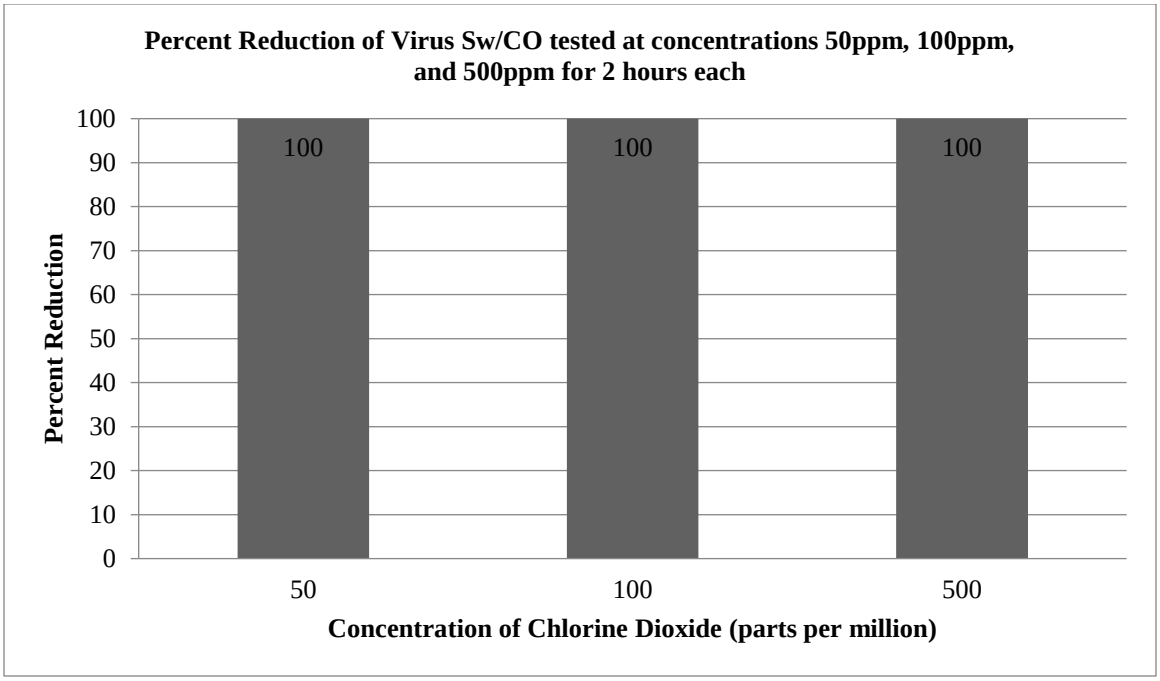

Figure 18: Calculated percent reduction of human virus Sw/CO/1/77 *tested at concentrations 50 ppm, 100 ppm, and 500 ppm for 2 hours each.*

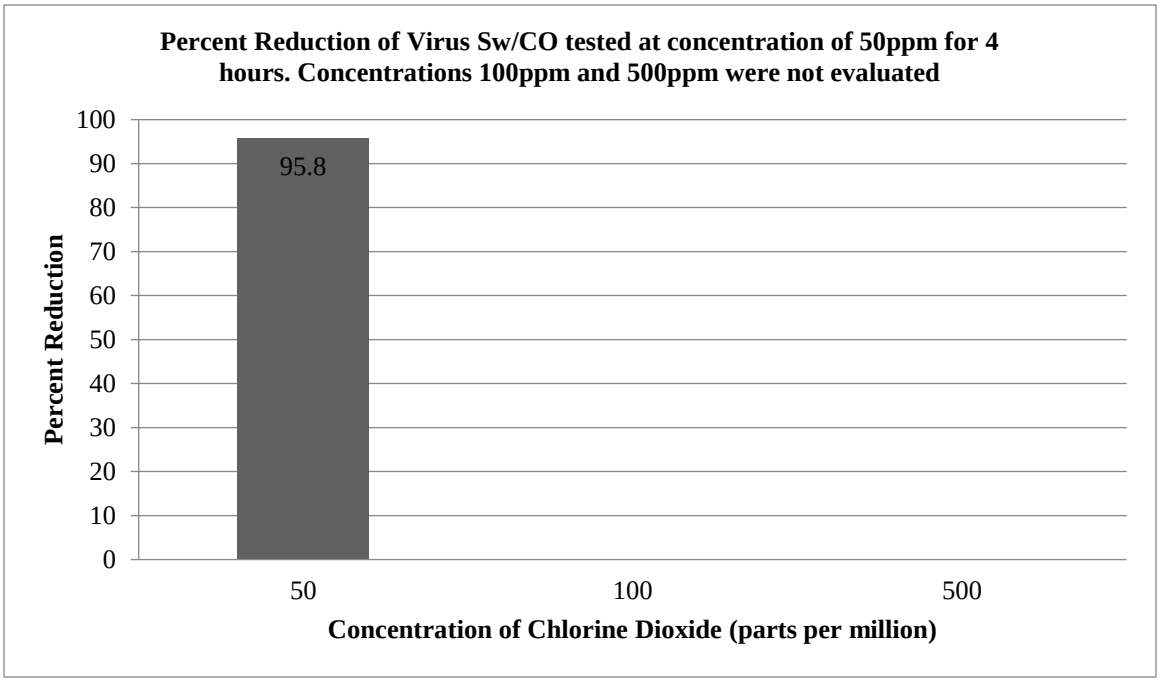

Figure 19: Calculated percent reduction of human virus Sw/CO/1/77 *tested at concentrations 50 ppm for 4 hours. Concentrations of 100 ppm and 500 ppm were not evaluated at this time length because 100% reduction was nearly achieved at 50 ppm.*

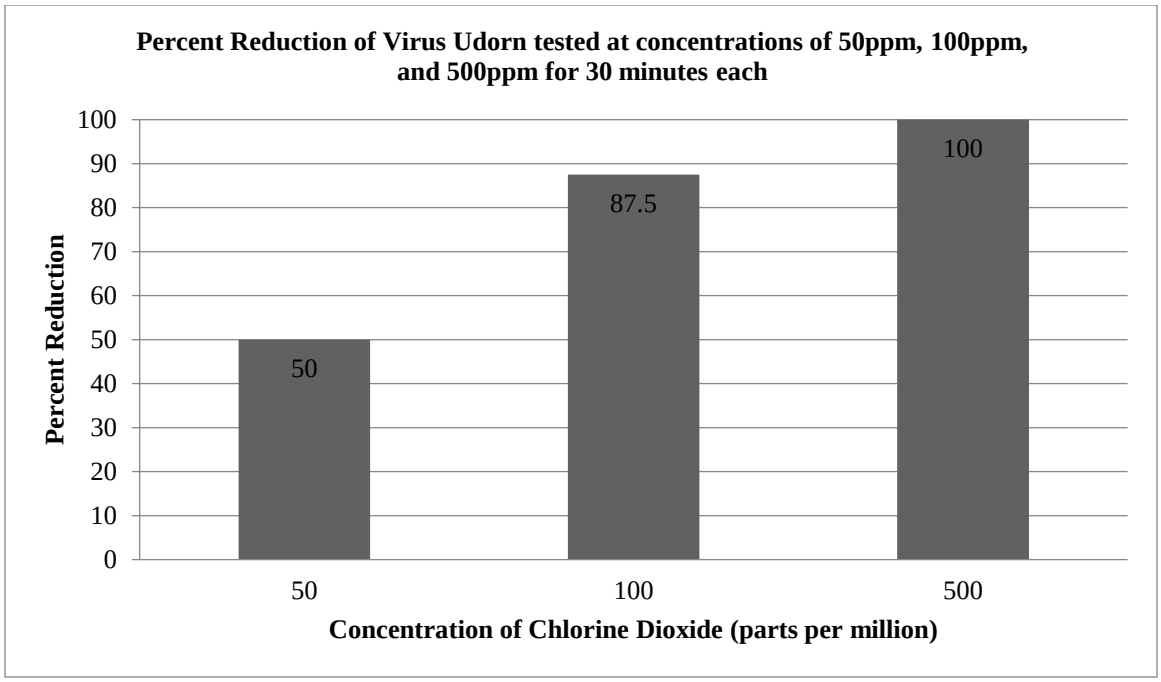

Figure 20: Calculated percent reduction of human virus Udorn/307/72 *tested at concentrations 50 ppm, 100 ppm, and 500 ppm for 30 minutes each.*

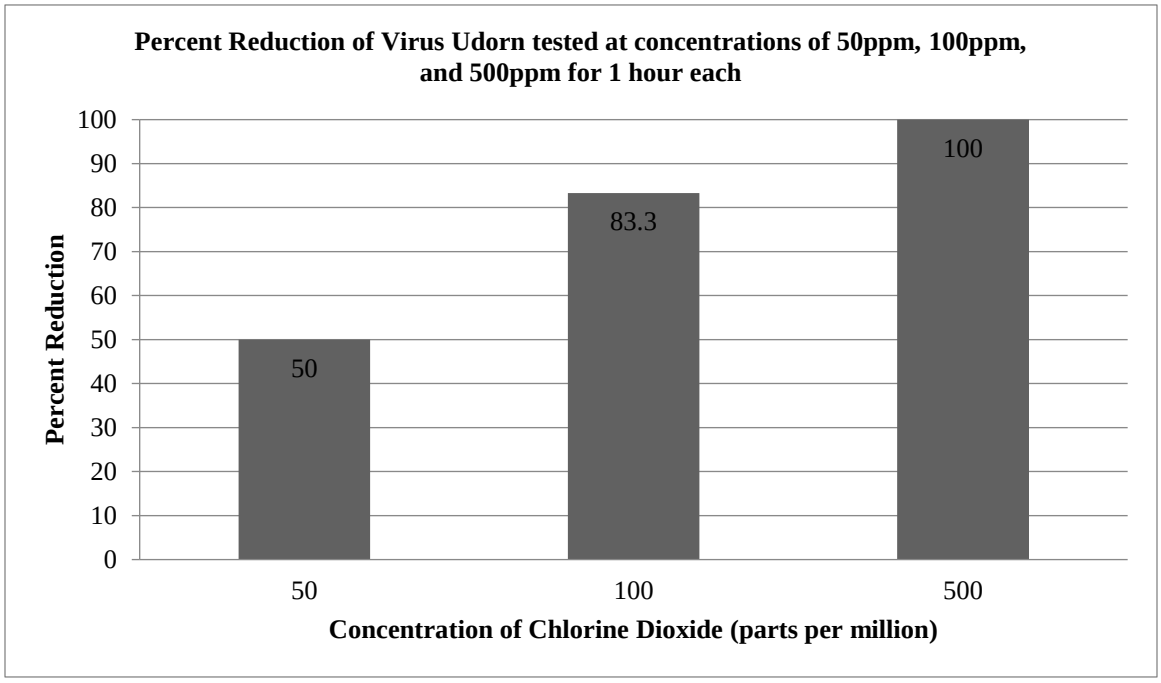

Figure 21: Calculated percent reduction of human virus Udorn/307/72 *tested at concentrations 50 ppm, 100 ppm, and 500 ppm for 1 hour each.*

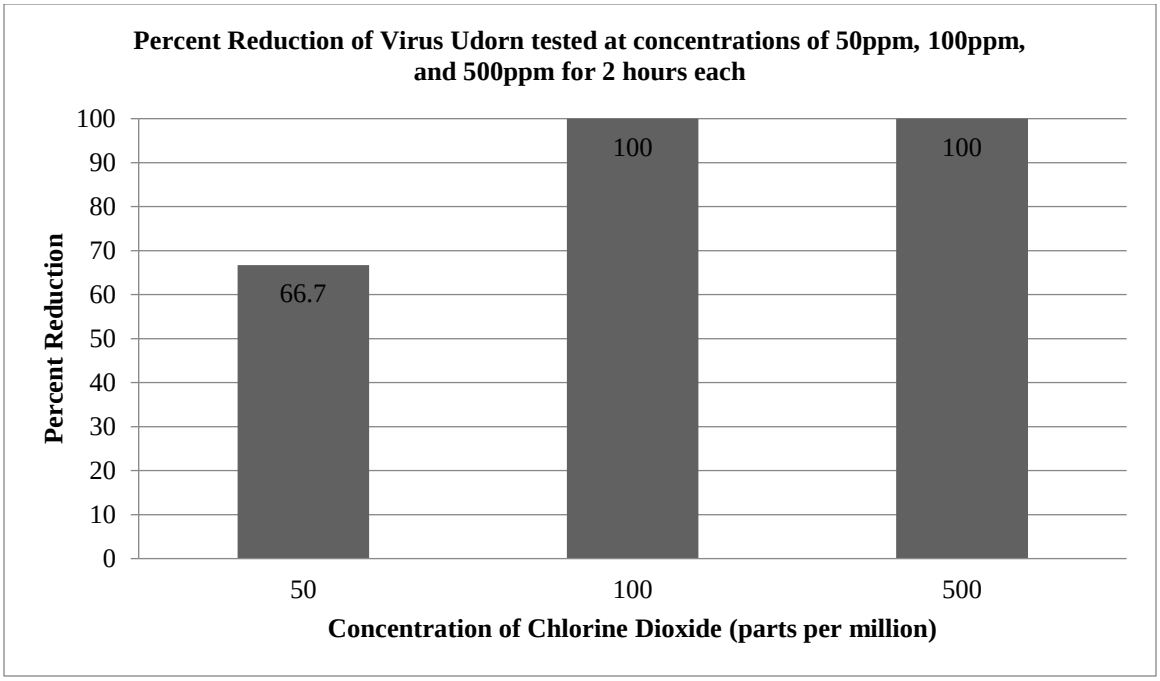

Figure 22: Calculated percent reduction of human virus Udorn/307/72 *tested at concentrations 50 ppm, 100 ppm, and 500 ppm for 2 hours each.*

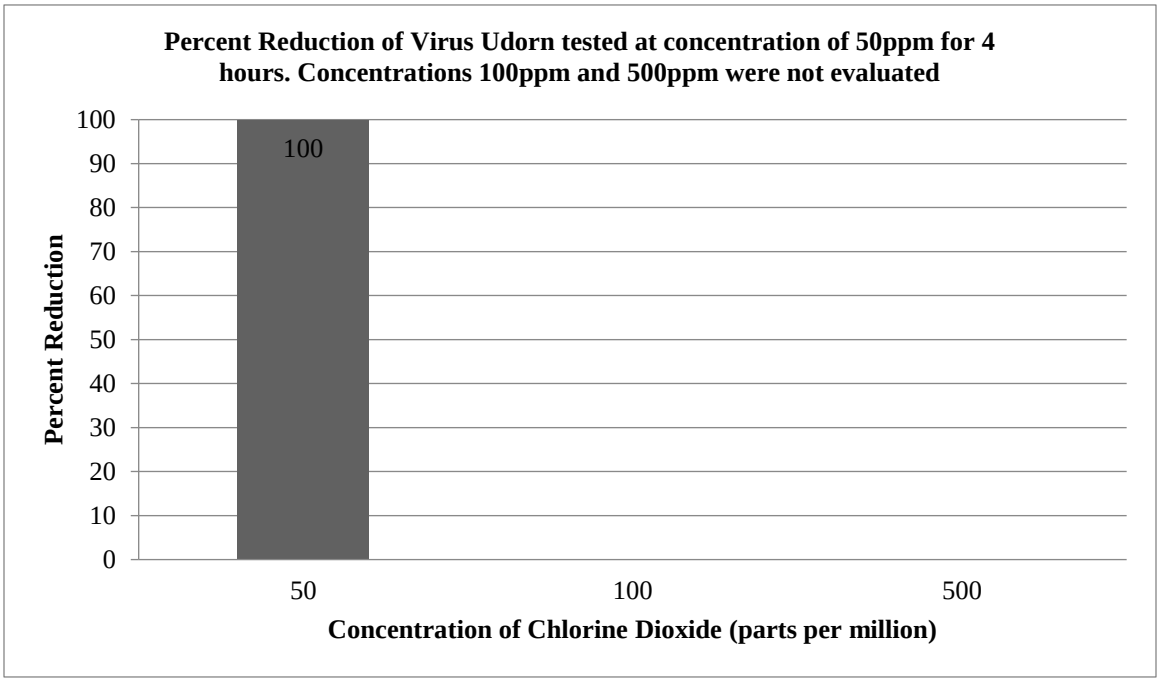

Figure 23: Calculated percent reduction of human virus Udorn/307/72 *tested at concentrations 50 ppm for 4 hours. Concentrations of 100 ppm and 500 ppm were not evaluated at this time length because 100% reduction was achieved at 50 ppm.*

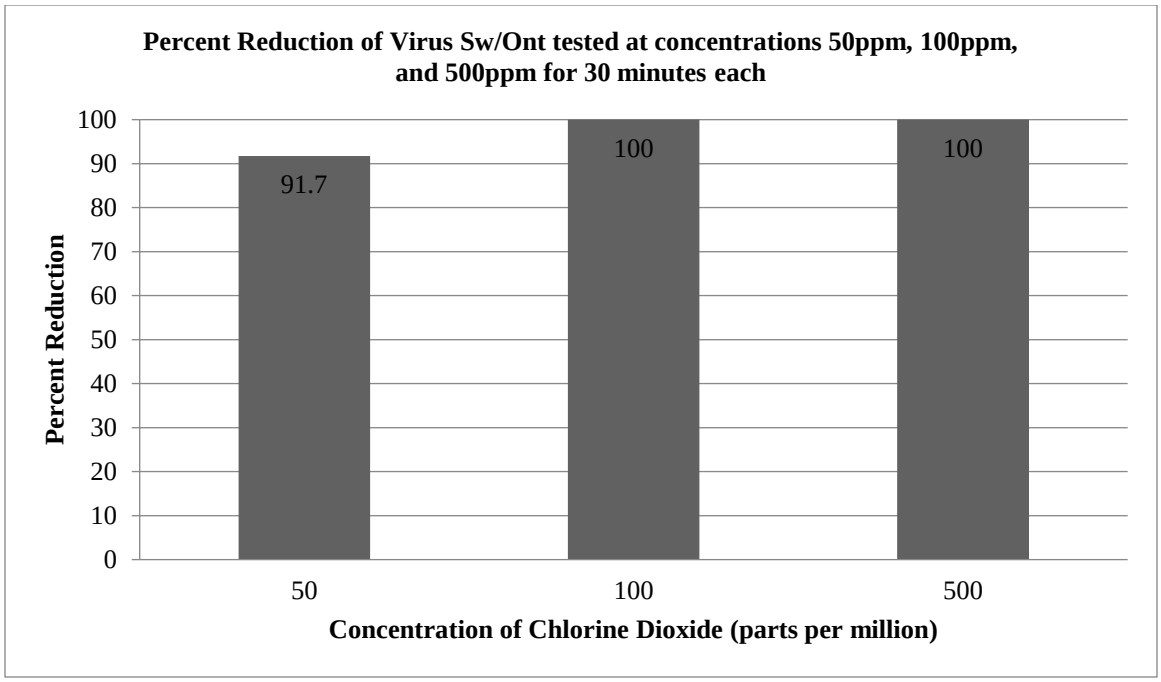

Figure 24: Calculated percent reduction of swine virus Sw/Ont/2/81 *tested at concentrations 50 ppm, 100 ppm, and 500 ppm for 30 minutes each.*

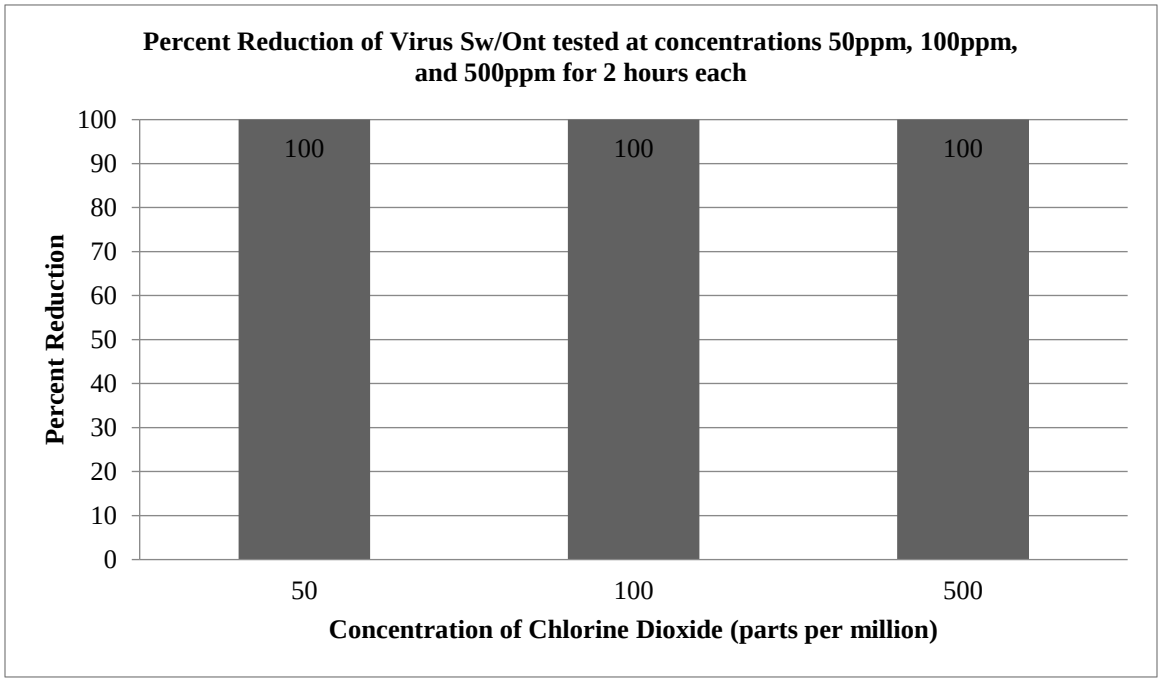

Figure 25: Calculated percent reduction of swine virus Sw/Ont/2/81 *tested at concentrations 50 ppm, 100 ppm, and 500 ppm for 1 hour each.*

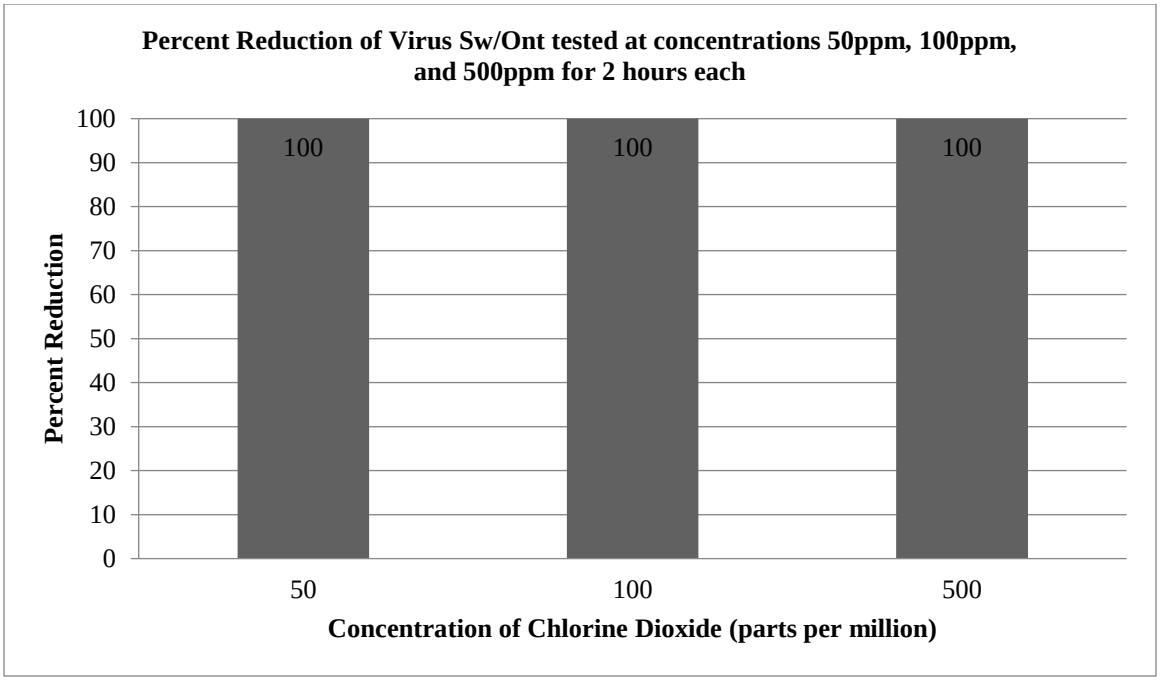

Figure 26: Calculated percent reduction of swine virus Sw/Ont/2/81 *tested at concentrations 50 ppm, 100 ppm, and 500 ppm for 2 hours each.*

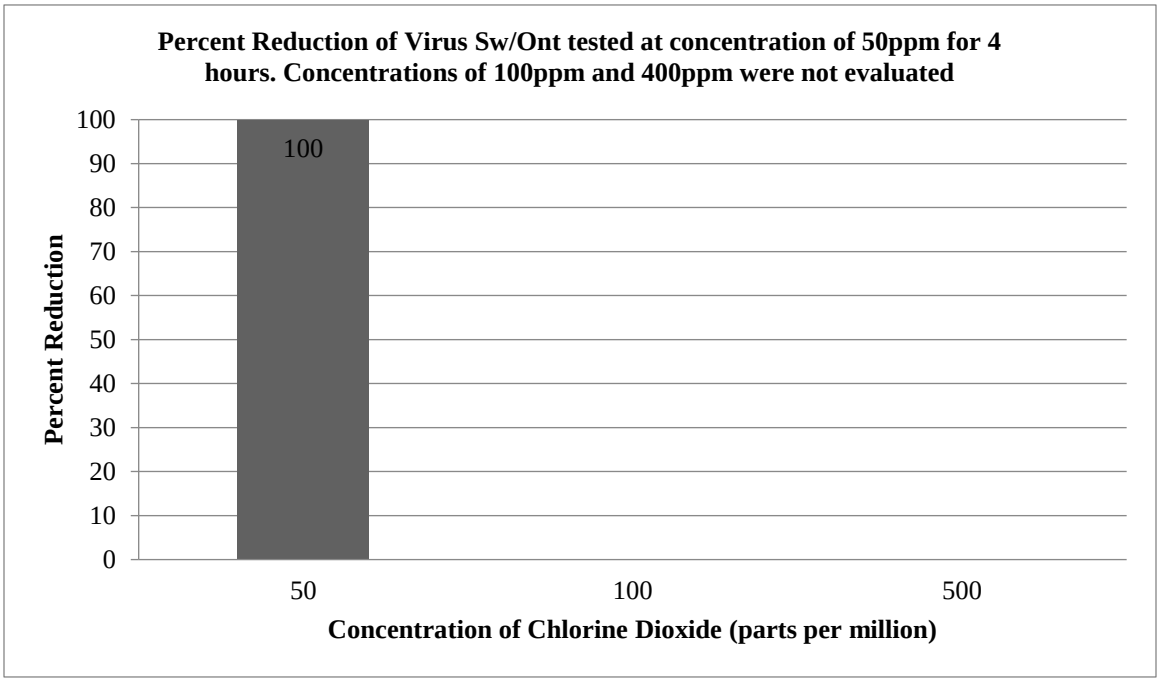

Figure 27: Calculated percent reduction of swine virus Sw/Ont/2/81 *tested at concentrations 50 ppm for 4 hours. Concentrations of 100 ppm and 500 ppm were not evaluated at this time length because 100% reduction was achieved at 50 ppm.*

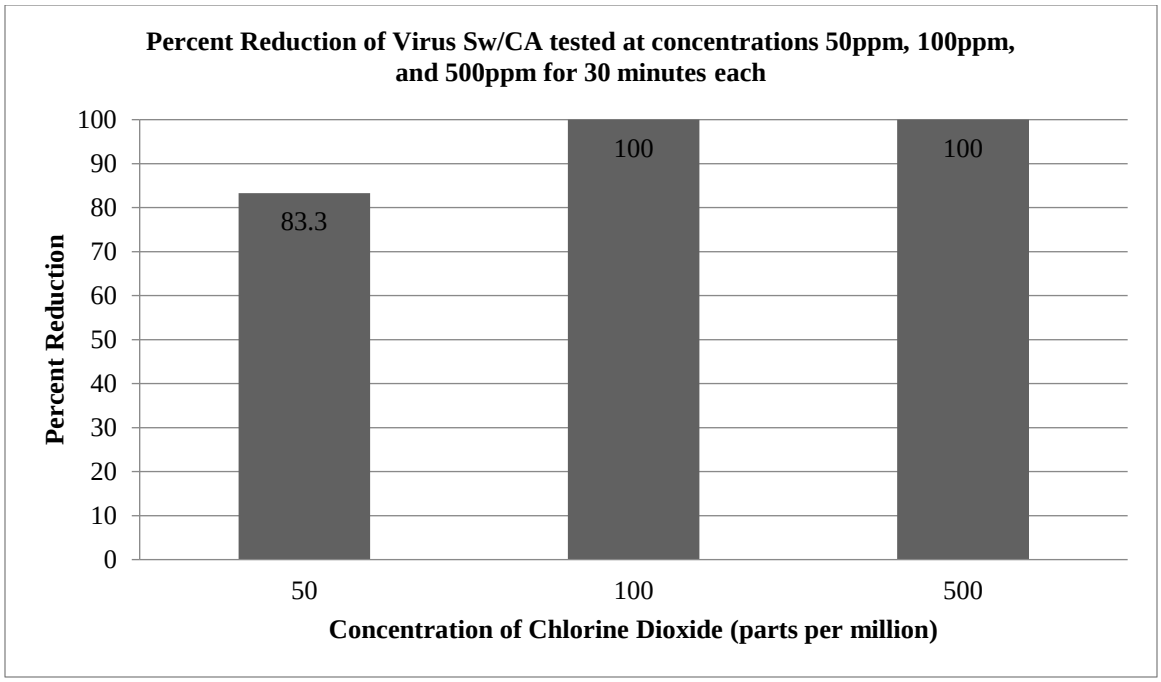

Figure 28: Calculated percent reduction of swine virus Sw/CA/90 *tested at concentrations 50 ppm, 100 ppm, and 500 ppm for 30 minutes each.*

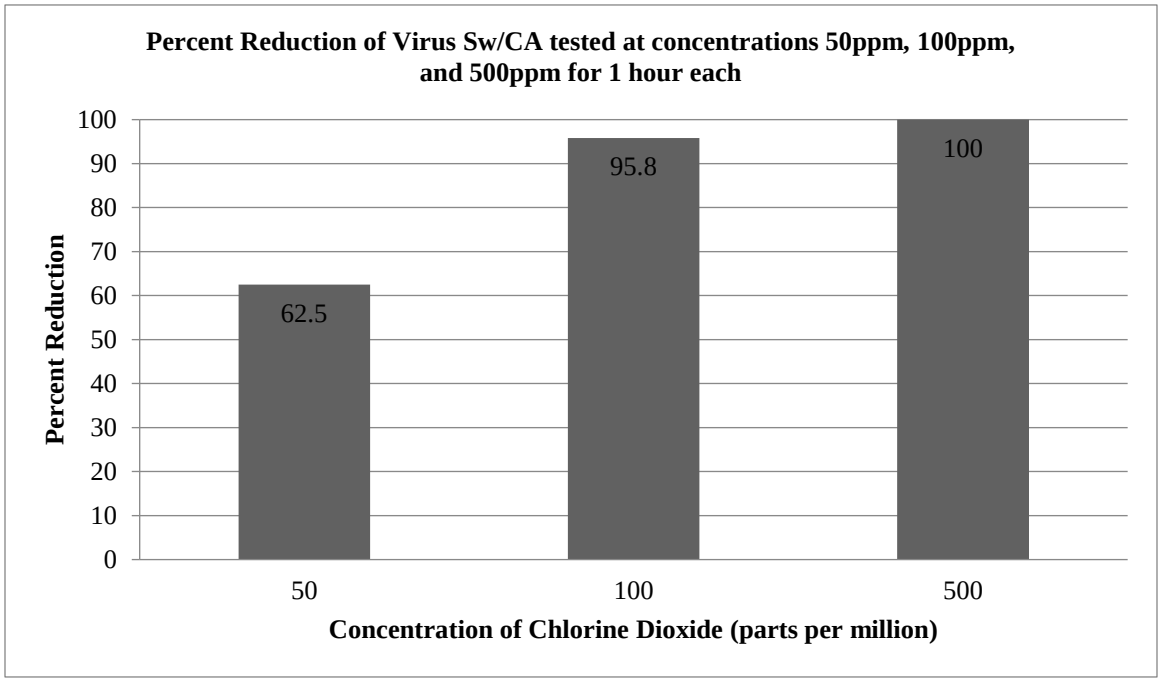

Figure 29: Calculated percent reduction of swine virus Sw/CA/90 *tested at concentrations 50 ppm, 100 ppm, and 500 ppm for 1 hour each.*

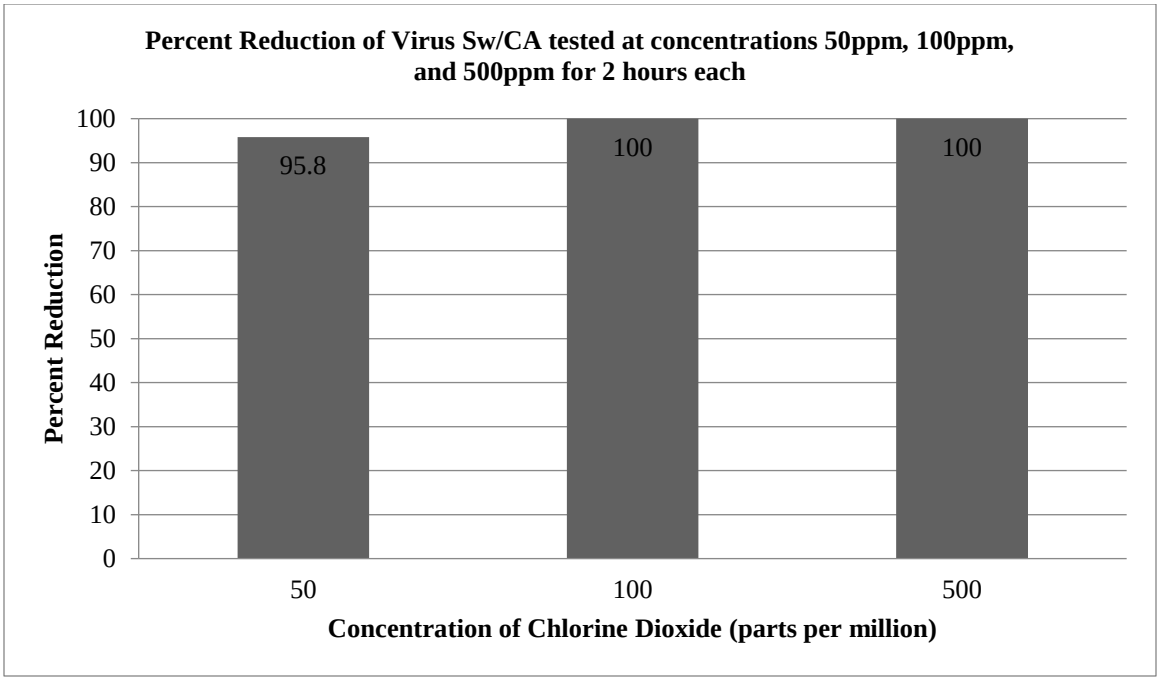

Figure 30: Calculated percent reduction of swine virus Sw/CA/90 *tested at concentrations 50 ppm, 100 ppm, and 500 ppm for 2 hours each.*

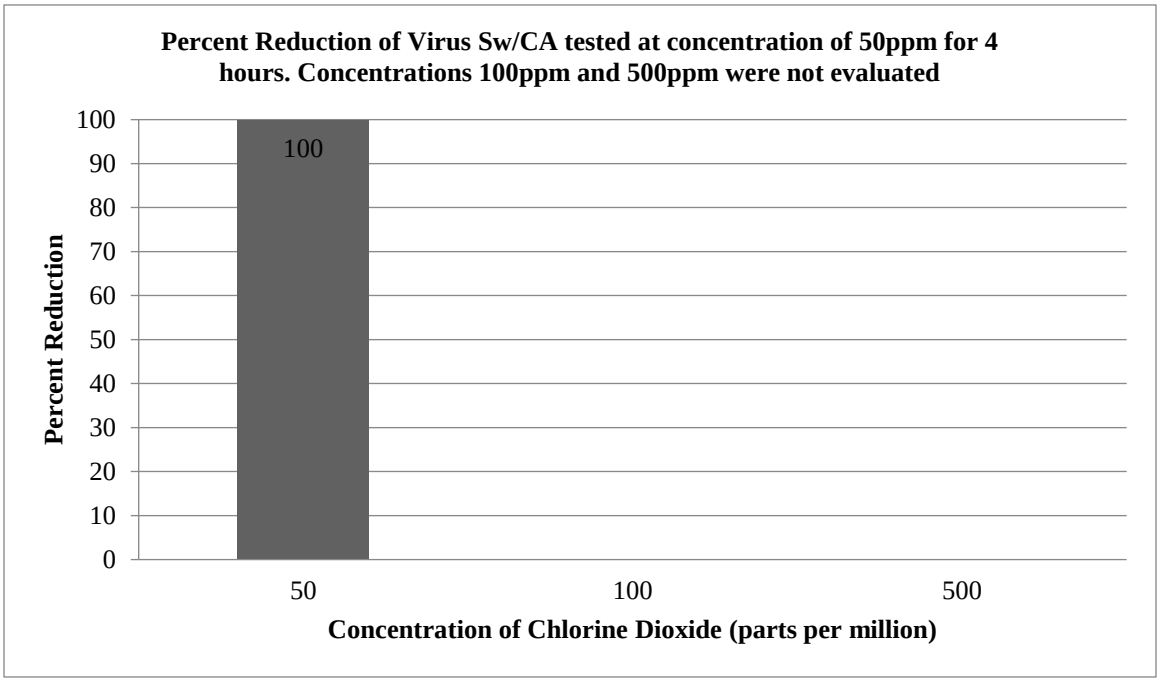

Figure 31: Calculated percent reduction of swine virus Sw/CA/90 *tested at concentrations 50 ppm for 4 hours. Concentrations of 100 ppm and 500 ppm were not evaluated at this time length because 100% reduction was achieved at 50 ppm.*

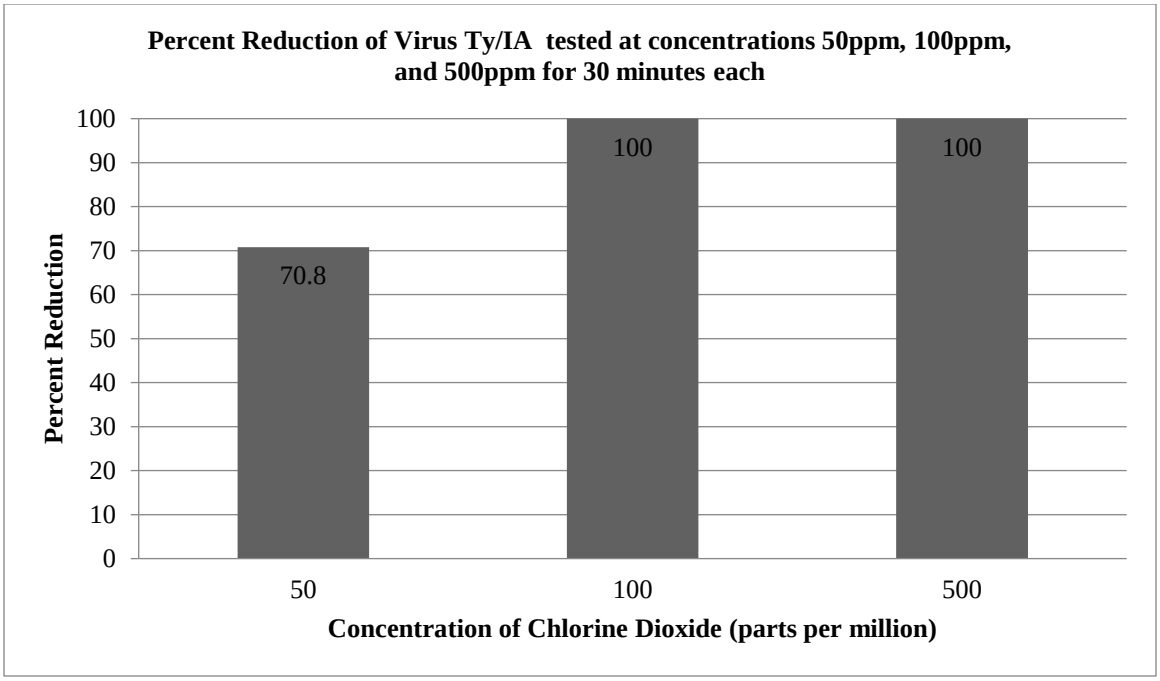

Figure 32: Calculated percent reduction of swine virus Ty/IA/7352/80 *tested at concentrations 50 ppm, 100 ppm, and 500 ppm for 30 minutes each.*

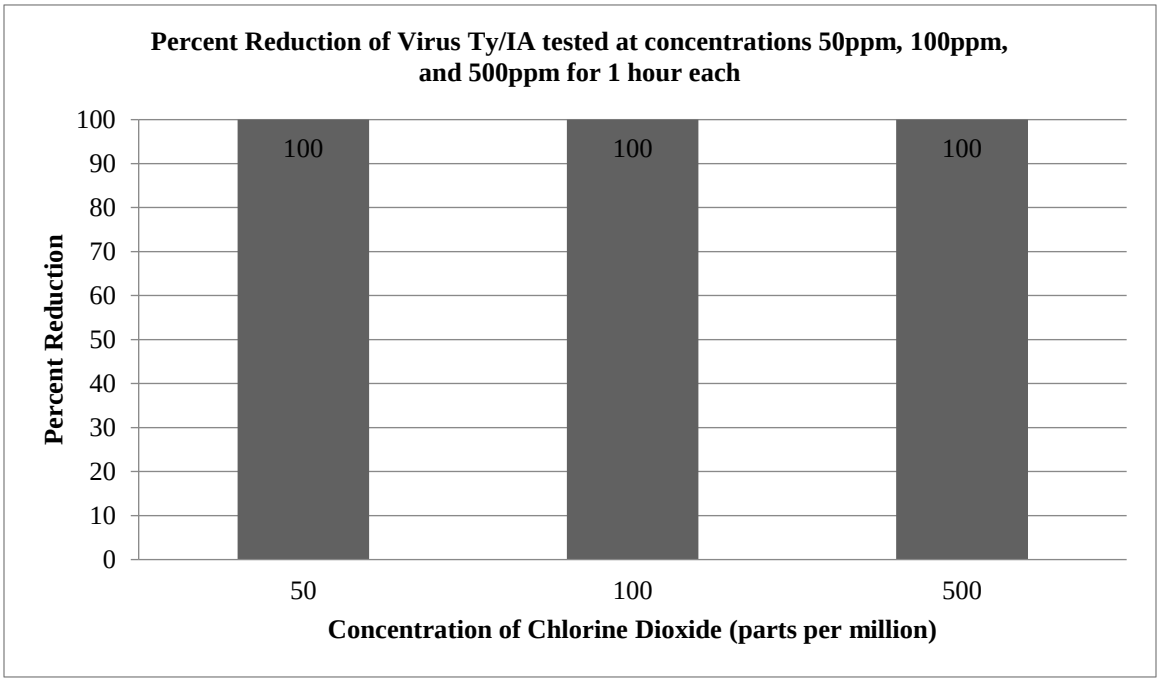

Figure 33: Calculated percent reduction of swine virus Ty/IA/7352/80 *tested at concentrations 50 ppm, 100 ppm, and 500 ppm for 1 hour each.*

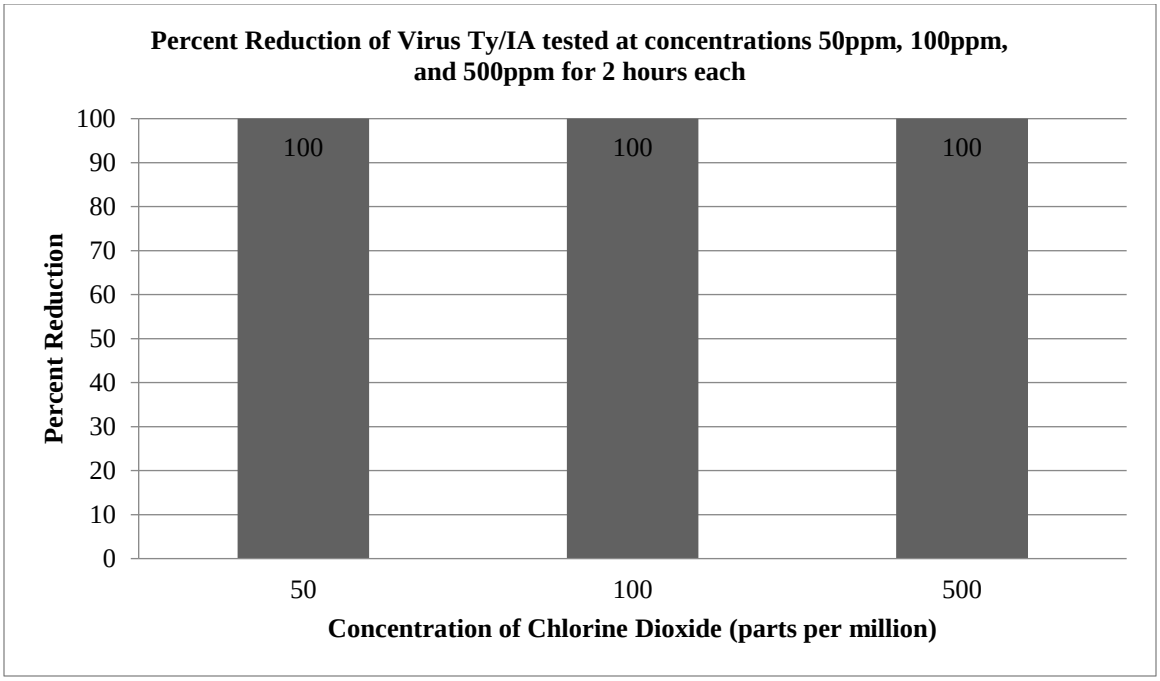

Figure 34: Calculated percent reduction of swine virus Ty/IA/7352/80 *tested at concentrations 50 ppm, 100 ppm, and 500 ppm for 2 hours each.*

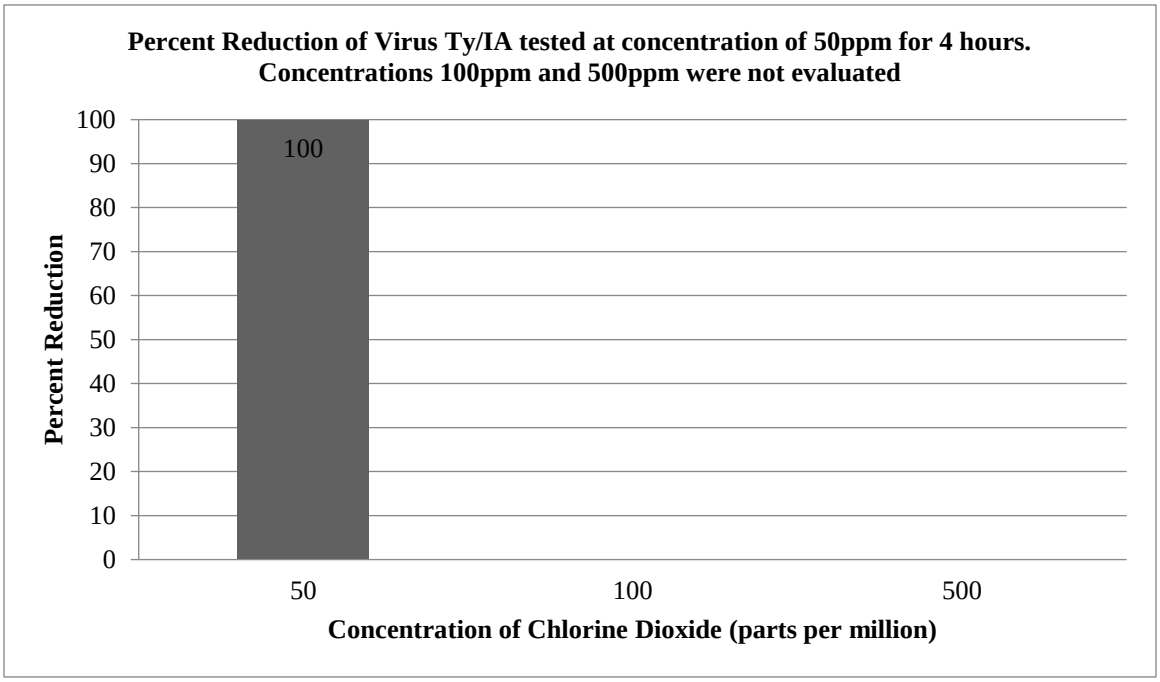

Figure 35: Calculated percent reduction of swine virus Ty/IA/7352/80 *tested at concentrations 50 ppm for 4 hours. Concentrations of 100 ppm and 500 ppm were not evaluated at this time length because 100% reduction was achieved at 50 ppm.*

DISCUSSION

Influenza viruses may be highly pathogenic, resulting in high rates of mortality and high rates of infection. Influenza viruses have a special advantage in that they can acquire a large range of variation by alterations of their cell surface proteins, particularly the hemagglutinin. This has led to widespread, devastating outbreaks that have occurred across the country on poultry farms. Besides the concern for the potential effects the diseases could have on humans and the possibility for avian to human transmission, the greatest toll currently that these viruses are taking are on the farming industries. Commercial farms of turkeys and chickens are losing their product and profits to the infection because of the high rate of speed that infection can take over entire flocks. This affects the everyday American consumer as prices of eggs, chicken meat, and other products rise dramatically. These outbreaks have cost Federal taxpayers over $950 million (USDA, 2016). On a broader scale, international trade is being blocked on certain products (USDA, 2015).

Influenza viruses have been studied a great deal, so the lack of knowledge on how to combat these outbreaks is not necessarily the problem - it is the mechanism. Vaccines have been developed that are administered every fall in order to protect humans from a potential flu virus, and there are therapeutic treatments known that can actually kill the virus (Webster and Govorkova, 2014). The problem arises when there is a mass spread of the virus, and no efficient or cost effective way to resolve the issues, as evident with HPAI in poultry. The most common method to get rid of the avian virus is to kill the host, and that leads to the euthanizing of entire flocks, thousands of birds at a time. Chlorine

dioxide has long been known to have antiviral activity against infectious agents (Ogata and Takahsi, 2008). So, this study used that knowledge to determine just how effective the chlorine dioxide gas could be on different influenza viruses over varying times and concentrations.

This study considered many aspects that would help gain an understanding of how chlorine dioxide can be used as a preventative treatment without harming poultry. This study included seven different virus strains, including two avian strains, two human strains, and three swine strains. Each virus received the same treatments. The treatments were based on exposure time and concentration of chlorine dioxide, and each treatment was repeated three times. The repetition of trials was important to our study, because it took into account variation that could occur with the mixtures of the granular components of chlorine dioxide. Especially at the low concentrations, the amounts of each A and B component were very small. The provider of the fast release chlorine dioxide product advised that these small amounts needed to be carefully placed to ensure good contact, that would ensure a good release (Joel Tenney ICA TriNova, personal communication, 2016 February 2). This was carried out as best as possible, but some of the variation could have been due to this factor. Errors in pipetting when conducting the hemagglutination assay also could have led to variation in results. When working with small amounts of virus, any small excess could offset the titer.

These results can only definitively be applied to the specific strains that were tested. However, the results are promising considering the range of viruses we tested, especially considering the different hosts. This study is important because it hopes to find

a treatment that could be applied to the current outbreaks and prevent the infection and transmission of influenza viruses among poultry.

There have been treatments using chlorine dioxide, but challenges and inconveniences prevent it from being an operative treatment. The method we used to introduce the chlorine dioxide eliminates these factors and allows the treatment to be more successful. As stated before, the product is a two-part system, consisting of components “A” and “B”. These are mixed in equal parts, and an oxidizing reaction allows the release of the chlorine dioxide gas from the granules. This method would be much more convenient than the unstable gas form treatments used previously. In general, the method of application to actual farms would be to spread the granules under the floor where the chickens stand. The reason for this relates to the method of transmission of the viruses, which is through the excrement of the birds. In this location, the chlorine dioxide would most likely be most effective. Although chlorine dioxide is toxic at high exposures over a long period, levels of that intensity are not required for the chemical to be effective. At low enough concentrations, the treatment could be applied while the chickens are present, which could be an advantageous ongoing preventative treatment.

Other literature reports that chlorine dioxide can be effective against a wide variety of infectious agents, including bacteria and viruses. Influenza A virus strains New Caledonia/20/99 and PR/8/34 are examples of other strains that have been tested and shown susceptibility to chlorine dioxide (Ogata and Takashi, 2008). These viruses were exposed to mice in aerosolized form while there was a low concentration of chlorine dioxide gas present, and the results showed higher concentrations of virus in the mice that were not exposed to the gas than the ones that were exposed (Ogata and Takashi, 2008).

Our results also showed a decreased concentration of virus after samples had been treated with chlorine dioxide gas, both of these results due to the chemical's ability to interact and change the conformation of the hemagglutinin protein before it could become infectious. Although avian influenza viruses are spread through fecal matter, we would hope to see the same preventative effects if a similar technique was implemented in chicken cages.

Chlorine dioxide was also tested against feline calicivirus, human influenza virus, measles virus, canine distemper virus, human herpesvirus, human adenovirus, canine adenovirus, and canine parvovirus in a study comparing two antiviral agents (Sanekata et al., 2010). The study included concentrations from 1 ppm to 100 ppm at 15 seconds of exposure, and this resulted in more than 99.9% inactivation of the viruses (Sanekata et al., 2010). Like our experiments, this study included many types of viruses, including human influenza virus, and reinforced our findings that chlorine dioxide has the ability to inactivate many different types and subtypes of virus.

Other viruses shown to be affected by chlorine dioxide are types H1N1 and H5N1 (Lénès et al., 2010). In this study, these viruses were introduced to water samples and chlorine dioxide treatments were added to the water (Lénès et al., 2010). This study found that chlorine dioxide was completely successful in inactivating the viruses, even after the lowest time implemented, 5 minutes (Lénès et al., 2010). One of the subtypes used in this experiment was H1N1, and this matches four of the subtypes used for our trials, including one avian strain and three swine strains.

Chlorine dioxide gas has the ability eliminate not only viruses but also bacteria commonly found on the skin, as well as *Bacillus* spores (Stubblefield and Newsome,

2015). This study implemented similar techniques of chlorine dioxide release used to test the influenza viruses in our experiments. This method allows chlorine dioxide to be generated without a power source, and provides higher stability and a longer shelf life, all which are advantageous if used on chicken farms (Stubblefield and Newsome, 2015).

Our study and results supported many different sources in stating that chlorine dioxide has high antiviral activity, especially against type A viruses. This reinforces the conclusion that chlorine dioxide has the potential to be a highly effective and efficient way to disable influenza viruses and prevent the future spread of the recent outbreaks.

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