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MRSA eradication using chlorine dioxide

Abstract

Antimicrobial-resistant (AMR) infections currently claim at least 50,000 lives each year across Europe and the US alone, with many hundreds of thousands more dying in other areas of the world. In 15 European countries, more than 10% of bloodstream Staphylococcus aureus infections are caused by methicillin-resistant strains (MRSA), with several of these countries seeing resistance rates closer to 50%.[1] Moreover, while the number of antibiotic-resistant infections is on the rise, the number of new antibiotics is declining.[1,2] It is therefore imperative that new, novel treatments of AMR's are sought, and this is the premise of this research – using natural substances to eradicate MRSA, that do not create further resistance. Chlorine dioxide used in vitro, has been our main focus of this research, as it was the most effective, compared to other natural substances tested.

Keywords: antimicrobial-resistant, methicillin-resistant strains, staphylococcus aureu, toxic shock syndrome, erythromycin, chlorine dioxide

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Abbreviations: MRSA, methicillin-resistant staphylococcus aureus; AMR. antimicrobial-resistant; TSST-1, toxic shock syndrome toxin–1; ClO_2, chlorine dioxide, PVL, panton-valentine leucocidin; MSSA, methicillin-susceptible saphylococcus aureus

Introduction

Frequently, Nosocomial Infections acquired in hospitals or ICUs, are caused by antibiotic-resistant bacteria such as Methicillin-Resistant Staphylococcus Aureus (MRSA). This antibiotic resistance is accompanied by high rates of morbidity, mortality, and the high cost of health care facilities.

What is MRSA?

Staphylococcus aureus is a gram-positive coccus that is both catalase- and coagulase+. Staphylococcus aureus has evolved to develop numerous immune evasion strategies to combat neutrophil-mediated killing, such as neutrophil activation, migration to the site of infection, bacterial opsonization, phagocytosis, and subsequent neutrophil-mediated killing. As many as 40 immune-evasion molecules of S. aureus are known, and new functions are being identified for these evasion proteins.

They produce a range of toxins, including alpha-toxin, beta-toxin, gamma-toxin, delta-toxin, exfoliatin, enterotoxins, Panton-Valentine leukocidin (PVL), and toxic shock syndrome toxin–1 (TSST-1); enterotoxins and TSST-1 are associated with toxic shock syndrome; PVL is associated with necrotic skin and lung infections and is a major virulence factor for pneumonia and osteomyelitis.[3]

S. aureus expresses a wide range of virulence factors, including toxins (haemolysins and leukocidins), immune-evasive surface factors (for example, capsule and protein A), and enzymes that promote tissue invasion (for example, hyaluronidase).[3]

MRSA colonization increases the risk of infection, and infecting strains match colonizing strains in as many as 50–80% of cases.[4,5] Nearly any item in contact with skin can serve as a fomite in MRSA transmission, from white coats and ties to pens and mobile telephones.

Colonization can persist for long periods. MRSA may also persist within the home environment, complicating attempts of eradication.[6] At the same time, colonization is not static, as strains have been found to evolve and even to be replaced within the same host.[7]

Drug resistance

MGEs carrying antibiotic resistance genes have been acquired by MRSA on multiple independent occasions. Resistance to penicillin (blaZ), trimethoprim (dfrA and dfrK), erythromycin (ermC), clindamycin (constitutively expressed ermC) and tetracyclines (tetK and tetL) have all been identified on insertion sequences, transposons and sometimes plasmids in both MRSA and methicillin-susceptible Staphylococcus aureus (MSSA).[8] Likely reflecting the strong selective pressures within the hospital environment, antibiotic resistance is often genetically linked to disinfectant or heavy metal resistance (for example, quaternary ammonia compounds, mercury or cadmium) among HA-MRSA strains.[9]

What is chlorine dioxide

The compound chlorine dioxide (ClO_2), now commercially important, is not a recent discovery. The gas was first produced by Humphrey Davy in 1811 when reacting hydrochloric acid with potassium chlorate. This yielded "euchlorine", as it was then termed. Watt and Burgess, who invented alkaline pulp bleaching in 1834, mentioned euchlorine as a bleaching agent in their first patent.[10,11]

Chlorine dioxide then became well known as bleach and later a disinfectant. The production of ClO_2 from the mineral chlorate is complicated, however, and the gas is explosive so that it could not be easily utilized practically until the production of sodium chlorite powder by Olin Corporation in 1940.

Chlorine dioxide could now be released when necessary from the chlorite salt. In municipal water supplies this is usually done by adding chlorine to the chlorite solution, and in the laboratory by adding an acid to the chlorite solution. Alliger showed in 1978,[10,11] that ClO_2 could be applied topically by the individual user.

ClO_2 is a small molecule with a molecular weight of 67.46, and it forms a stable radical.[12] ClO_2 is an oxidizer, which is reduced to chlorite ion (ClO_2 -) by capturing an electron (ClO_2 + e- → ClO_2 -). The redox potential (E^o) is relatively high as 0.95 V, therefore does not harm the human microbiome.[13,14]

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Chlorine dioxide (ClO2) solution

Chlorine dioxide is: bactericidal, virucidal, sporicidal, cysticidal, algicidal, and fungicidal.[15] It has been reported that chlorine dioxide, a strong oxidant, can inhibit or destroy microorganisms at concentrations ranging from 1 to 100 ppm which produced potent antiviral activity, inactivating >or= 99.9% of the viruses with a 15-sec treatment for sensitization.[15-19]

Moreover, ClO_2 can remove biofilms swiftly[20] because it is highly soluble in water and unlike ozone, it does not react with the extracellular polysaccharides of the biofilm. This way ClO_2 can penetrate biofilms rapidly to reach and kill the microbes living within the film – a huge advantage that is different to tackle for both Natural and Allopathic Medicine. There are many reports that ClO_2 solution has a virucidal activity.[21-25] The inactivation concentration against various viruses is 1-2ppm in poliovirus.[21,22] 2.19ppm in coronavirus which causes SARS.[23] 7.5ppm in hepatitis A virus,[24] and 0.2ppm in rotavirus.[25]

Safety of chlorine dioxide

Many evaluations have shown ClO_2 compounds to be non-toxic. Five decades of use have not indicated any adverse effects on health. The main areas of use have been disinfecting water supplies, the elimination of unwanted tastes and odours, and bleaching in the pulp and paper, and textile industries.

Toxicology tests include ingestion of ClO_2 in drinking water, additions to tissue culture, injections into the blood, seed disinfection,[26,27] insect egg disinfection, injections under the skin of animals and into the brains of mice, burns administered to over 1500 rats, and injections into the stalks of plants. Standard tests include Ames Mutation, Chinese Hamster, Rabbits Eye, Skin Abrasion, Pharmacodynamics and Teratology.[28]

In one study, human volunteers drank ClO_2 or ClO_2^- in solution up to 24 ppm and showed no adverse effects.[28]

Several studies examined the effects on reproductive toxicity or teratology. There is no evidence of fetal malformation or birth defects at ClO_2 concentrations, in drinking as well as skin route, up to 100ppm.[29-31]

With prolonged feeding toxicity is produced mainly in the red blood cell. Rats fed up to 1000mg/l chronically for 6 months showed no significant hematological changes. After 9 months, however, red blood cell counts, hematocrit, and hemoglobin were decreased in all treatment groups.

Lack of toxicity in the long term, but the low-level basis is dramatically illustrated by two separate studies where rats[32] and honeybees[33] were fed ClO2 in high doses over two years. No ill effects were noted with up to 100 ppm added to the water supply.

Materials and methods

Methicillin-resistant Staphylococcus Aureus (MRSA) was used in this research study, grown on blood, agar plates, which were provided by a local clinical laboratory with certification.

Culturing MRSA

In a Safety Class 2 cabinet, from the Blood agar plates (Columbian Agar), a sample of MRSA from isolated cultures was taken using a sterilized loop and placed in sterile tubes with 5 ml of Tryptic Soy Broth (TSB). These culture tubes were incubated at 37 degrees centigrade for 48 hours. These culture tubes could be stored in a refrigerator at 4 degrees centigrade for up to 10 days, whereby new samples would be made.

Counting bacteria

One of the most common methods to quantify bacteria is counting colony-forming units (CFUs). This widely used method is simple, gives a good general idea of cell viability, and is sensitive even to low concentrations of bacteria.

A major disadvantage is that it takes days to get results that are estimations at best. One colony may arise from one or a thousand cells and sample preparation can vary from tech to tech, as well as each time, depending on sample conditions. For the sake of increased accuracy, in this research, the QUANTOM Tx Microbial Cell Counter was used from Logos Biosystems (logosbio.com). It is an image-based, automated cell counter that can identify and count individual bacterial cells in minutes.

The QUANTOM Tx automatically focuses on, captures, and analyzes multiple images of fluorescence-stained cells to detect bacterial cells with high sensitivity and accuracy. It contains a sophisticated cell detection and declustering algorithm that can accurately identify individual bacterial cells in even the tightest clusters. In these experiments, we used the Viable Cell Staining Kit to detect live or viable cells.

The Quantom Microbial Cell Counter has been compared and found to be as accurate as Flow Cytometry and Haemocytometer measurements, but greatly reducing the time as each count takes no longer than 30 seconds, and it can distinguish between clusters. Stained cells are mixed with QUANTOM Cell Loading Buffer I, loaded into QUANTOM M50 Cell Counting Slides, and spun in the QUANTOM Centrifuge to immobilize and evenly distribute the cells along a single focal plane to ensure accurate cell detection. Counting results and images can be viewed and saved immediately after the count.

To prepare the sample for the Quantom, 10 microlitres (ul) of the culture medium was taken using a DLAB electronic pipette that had been previously calibrated and placed in a 1.5ml sterilized Eppendorf tube. To this was added 2ul of Viable Cell Staining Dye and this was incubated in a Heraeus incubator at 37 degrees centigrade for 30 minutes. To this sample was added 8ul of Buffer to enhance the fluorescence signal. To save on the consumable Quantom slides, we recycled the slides by washing them in the iWash® Slide Cleaner Systems from Imrali Inventions (www.imraliinventions.com).

To these tubes, was added chlorine dioxide in different concentrations, for differing durations? The concentration of chlorine dioxide ranged from 0.5µl (0.5 ppm) to 5µl (5ppm), and the duration of exposure to the sample ranged from 30minutes to 30 seconds.

For each experiment based on time and duration, two tubes of the sample were prepared to keep the dilution factor constant. According to the amount of chlorine dioxide added to the experimental tube, the same quantity of water was added to the control tube.

From these Control and Experimental tubes, 6 µl of the sample was taken using an electronic pipette and placed on the M50 Cell Counting Slides. The slides were placed into the QUANTOM Centrifuge for 8mins at 300 RCF (Relative Centrifugal Force) and then placed into the Quantom Microbial Cell Counter to take a baseline measure (Control) and another measurement from the Experimental tube.

The optimum Quantom Microbial Cell Counter setting for the MRSA protocol that we found during testing was set to Dilution Factor 2, Minimum Fluorescence Object Size 0.4um, Maximum Fluorescent Object Size 15µm Roundness 50%, Declustering Level 7, and Detection Sensitivity 7.

Preparing Chlorine Dioxide

The traditional chlorine dioxide, called MMS, was prepared as a solution using two components, Sodium chlorite solution (25% solution in water) and Hydrochloric Acid 4% solution. One drop of each of these solutions was placed in a 1.5ml sterile Eppendorf tube and left for 30 seconds to activate. In addition, more experiments were performed using a new generation of chlorine dioxide called CDSplus, a patented product manufactured by Aquarius Pro-Life as a water treatment product. This is a buffered form of chlorine dioxide at a standard pH of 7 and a concentration of 3,000ppm when activated (250ml). From the activated CDSplus (250ml), was taken 83µl = 1ppm; 166 µl= 2ppm; 0.25ml = 3ppm.

Experimental Protocols

Several concentrations of chlorine dioxide – both the Traditional MMS and CDSplus were used. The range was from 1ppm - 5ppm. The time of exposure of the chlorine dioxide ranged from 30minutes down to 30 seconds. It was not clear in the initial experiments what time would be required for inhibition, but it was quickly demonstrated that it was less than one minute exposure. Most experiments, therefore, had an exposure time of 1minute.

Results

Initial Experiments

We began taking different chlorine dioxide concentrations based on the Traditional MMS and tested these concentrations with MRSA in solution for different times spanning from 30minutes to 30 seconds. 1µl of Chlorine dioxide is the equivalent of 1ppm concentration. The lowest concentration of Chlorine dioxide used to completely eradicate MRSA in these experiments was 0.5ppm, with an exposure time of 30 seconds. Table 1 below shows the different concentrations against time, with the MRSA cell concentration as measured by the Quantom Cell Counter. As can be seen, for all concentrations of chlorine dioxide ranging from 1 to 5ppm, and time of exposure from 30minutes down to 30 seconds, the growth inhibition of the MRSA was 99.99% throughout all these experiments.

Table 1 Comparison of Bacterial Counts Before and After Chlorine Dioxide Exposure

Time (Min)	Chlorine Dioxide Conc. (Ml = Ppm)	Cell Concentration		Average Size of Bacteria (µm)		Cell Number (Before & After)		Difference In Cell No	% Difference In Cell No
		C ($x\ 10^8$)	E ($x\ 10^6$)	C	E	C	E		
30	1 ppm	2.32	1.20	2.6	0.8	10012	52	9960	99.99
30	2 ppm	2.32	9.49	2.6	0.8	10012	41	9971	99.99
30	3 ppm	2.32	1.13	2.6	0.8	10012	49	9963	99.99
30	4 ppm	2.32	8.10	2.6	0.8	10012	35	9977	99.99
30	5 ppm	3.15	2.08	2.3	0.8	13591	9	13582	99.99
15	5 ppm	3.64	1.97	2.4	0.8	15177	85	15092	99.99
15	1 ppm	3.64	1.92	2.4	0.8	15177	83	15094	99.99
15	2 ppm	3.64	2.11	2.4	0.8	15177	91	15086	99.99
15	3 ppm	3.64	2.22	2.4	0.8	15177	96	15081	99.99
15	4 ppm	3.64	1.88	2.4	0.8	15177	81	15096	99.99
15	5 ppm	3.64	1.76	2.4	0.8	15177	76	15101	99.99
5	0.5 ppm	6.99	3.06	2.9	0.8	30200	132	30068	99.99
4	0.5 ppm	6.99	3.79	2.9	0.8	30200	156	30044	99.99
3	0.5 ppm	6.99	3.82	2.9	0.8	30200	165	30035	99.99
2	0.5 ppm	6.99	1.09	2.9	0.8	30200	47	30153	99.99
1	0.5 ppm	6.99	1.06	2.9	0.8	30200	46	30154	99.99
0.5	0.5 ppm	6.99	1.09	2.9	0.8	30200	47	30153	99.99

C, Control; E, Experimental

Experiment 1

From the initial experiments, given that Chlorine dioxide was found to eliminate 99.99% of the MRSA bacteria at concentrations of 0.5 ppm for only 30 seconds, all other experiments used a one-minute exposure time as standard, while testing different concentrations.

In this experiment, concentrations of ClO2 ranging from 0.5 – 5ppm were taken, using the Traditional MMS. In each of the 5 concentrations, the inhibition rate was 100% - see Table 2 and Figure 1. Figure 1 shows the repeatability of counting MRSA bacteria using different concentrations ranging from 1 - 5ppm. A baseline count was taken for each concentration – this was repeated 5 times. In all 5 repeats, the growth inhibition of the MRSA was 100%.

Table 2 shows the cell count numbers for the 6 concentrations of chlorine dioxide used, namely: 0.5, 1, 2, 3, 4, and 5 ppm were used and a baseline line count was measured for each concentration. Experiment number 0 is the baseline count (control) for each experiment group using different concentrations of chlorine dioxide. For each concentration, the experiment was repeated 5 times, with average concentrations given.

Figure 2 compares the cell count of MRSA cells against MMS concentration for 1 minute. The covered area is equal to the cell count number. The initial counts for each concentration are shown on the left side of the graph and the final is shown on the right side of the graph. The inhibition rate was 100% for all concentrations of chlorine dioxide, with an exposure time of 1 minute.

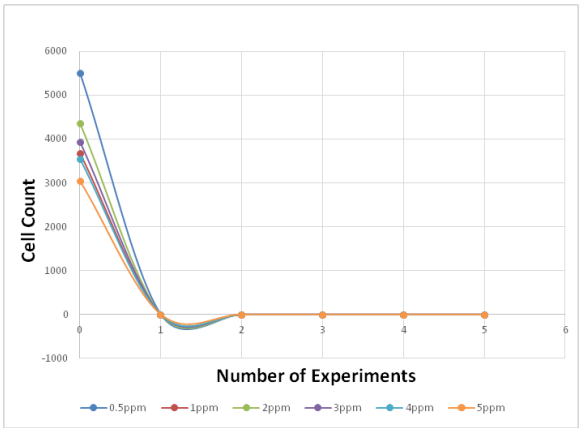

Figure 1 Chlorine Dioxide at Different Concentrations Using Traditional MMS.

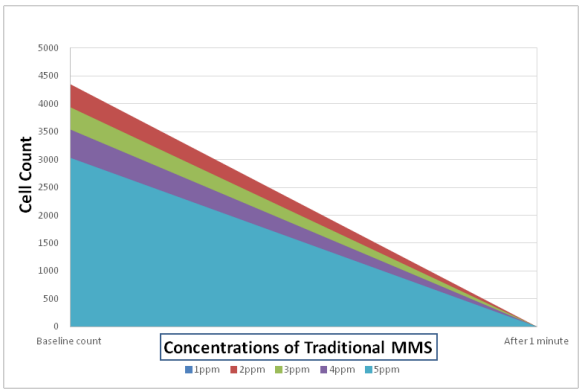

Figure 2 Different Concentrations of Traditional MMS for Duration of 1 Min.

Table 2 Chlorine Dioxide (Traditional MMS) at different concentrations repeated 5 times

Concentration of Chlorine dioxide	0.5ppm	1ppm	2ppm	3ppm	4ppm	5ppm
Experiment no.						
0 (baseline)	5502	3677	4360	3938	3542	3039
1	0	0	0	0	0	0
2	0	0	0	0	0	0
3	0	0	0	0	0	0
4	0	0	0	0	0	0
5	0	0	0	0	0	0

Table 3 compares the concentrations of 1, 2, 3, 4, and 5 ppm for 1-minute exposure to chlorine dioxide. The control was compared to the experimental for the different concentrations. For all these concentrations of chlorine dioxide, the inhibition rate was 100%.

Table 3 Chlorine Dioxide (Traditional MMS) at different concentrations for 1minute exposure

	1 PPM	2 PPM	3 PPM	4 PPM	5 PPM
Control	3677	4360	3938	3542	3039
Chlorine dioxide exposure for 1 min	0	0	0	0	0

Experiment 2 – using CDSplus

The same experiment as above was repeated using the CDSplus generation, using 1–3 ppm concentrations. In each of the 3 concentrations, the inhibition rate was again 100% - see table 4 and graph 3.

Figure 3 shows the eradication of MRSA cells using different concentrations of CDSplus, namely 1, 2, and 3ppm. A baseline count was measured for the control group, and then each concentration of CDSplus was added and repeated twice. For all concentrations, the inhibition rate was 100%. Table 4 compares the concentrations of 1, 2, and 3ppm for 60-second exposure to chlorine dioxide, using the new generation CDSplus. The control was compared to the experimental for the different concentrations.

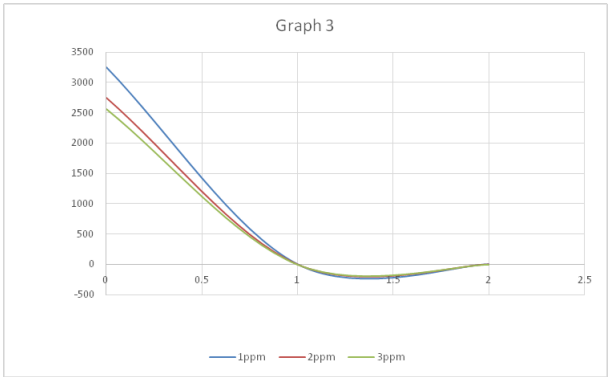

Figure 3 MRSA-CDSPlus with Different Concentrations.

Figure 4 compares the MRSA cell count against chlorine dioxide (CDS plus) concentration for 1 minute. The top line shows the baseline cell count for the control group. The bottom line shows the counts of MRSA cells after the exposure of the cells for 1 minute at different concentrations of chlorine dioxide – the inhibition rate was 100%.

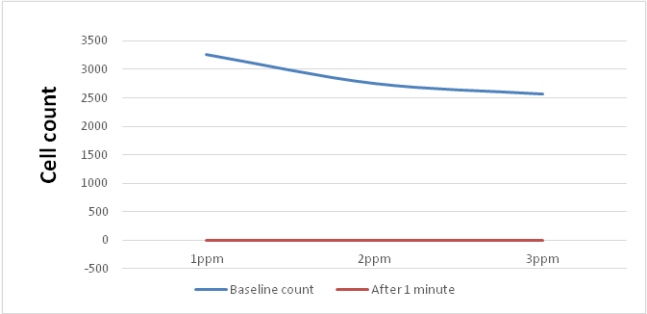

Figure 4 Different CDSPlus Concentration for 60 Seconds.

Table 4 Chlorine Dioxide (CDSplus) at different concentrations for 1 minute exposure

Concentration of Chlorine dioxide	1ppm	2ppm	3ppm
Experiment no.			
0 - Control	3256	2750	2565
1	1	0	0
2	1	0	0

Conclusions

MRSA is versatile, and unpredictable. Its capacity for genetic adaptation and the serial emergence of successful epidemic strains cause it to remain a major threat to human health.

The persistently high mortality associated with invasive MRSA infection — even though multiple antibiotics with effectiveness against MRSA have been approved by the FDA since 2014 — highlights the need for high-quality trials to determine optimal management for these patients. In these in vitro experiments, the efficacy of chlorine dioxide against MRSA has been shown consistently, with growth inhibition of 99.99% -100% in even the smallest concentrations of 0.5ppm.

Given the proven safety of chlorine dioxide in animal and human experiments to date, there is an urgent need for high-quality clinical trials to determine the efficacy of chlorine dioxide with individuals infected with MRSA today.

Such studies will fall upon the clinical community to conduct, beginning with individual clinical trials in different countries around the world, with the creation of a clinical trials network to collate all the data and develop safe and effective clinical protocols. Regarding safety, in one carefully designed experiment, it was found that the characteristic time necessary to kill a microbe is only a few milliseconds. As ClO_2 is a rather volatile compound its contact time (its staying on the treated surface) is limited to a few minutes.[36]

While this stay is safely long enough (being at least 3 orders of magnitude longer than the killing time) to inactivate all bacteria on the surface of the organism, it is too short for ClO_2 to penetrate deeper than a few tenths of a millimetre; thus, it cannot cause any real harm to an organism which is much larger than a bacterium.[36]

There are also many testimonials of chlorine dioxide being used by human volunteers for the eradication of many infectious diseases, including malaria and HIV, but one of the pioneers in Africa, Jim Humble. There is much controversy over this anecdotal evidence, but the number of witnesses giving testimonials cannot be ignored – politics and self-interests must be put aside and science must examine the evidence for the benefits of humanity![34,35]

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Conflicts of interest

Author declares there are no conflicts of interest.

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