RESEARCH ARTICLE

Eradication of Antibiotic-Resistant *E. coli, S. aureus, K. pneumoniae, S. pneumoniae, A. baumannii,* and *P. aeruginosa* with Chlorine Dioxide I*n Vitro*

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ABSTRACT
Bacterial Antibiotic Resistance (AMR) is a problem in all regions, with six pathogens accounting for 73.4% of deaths attributable to bacterial AMR, namely *Escherichia coli* (E. coli), *Staphylococcus aureus* (S. aureus), *Klebsiella pneumoniae* (K. pneumoniae), *Streptococcus pneumoniae* (S. pneumoniae), *Acinetobacter baumannii* (A baumannii), and *Psuedomonas aeruginosa* (P. aeruginosa). The World Health Organization instigated a Global Action Plan on AMR in 2021, which is still active - healthcare costs for AMR run into many billions of dollars worldwide. A Review on Antimicrobial Resistance commissioned by the British Government argued that AMR could kill 10 million people per year by 2050 and has emerged as one of the greatest public health threats of the 21st century. Just one AMR pathogen, *Methicillin-Resistant Staphylococcus aureus* (MRSA), caused more than 100,000 deaths worldwide, with the other four pathogens covered in this research causing as many deaths again. This research has focused on studying chlorine dioxide's effectiveness in eradicating five different AMR bacteria in vitro as a novel and effective treatment. This study used different chlorine dioxide concentrations with five antibiotic-resistant bacteria, ranging from 1 – 7 ppm concentrations. Disinfection studies were compared to controls, and the results demonstrated a greater than 95% disinfection with concentrations of 7 ppm. Chlorine dioxide is a size-selective antimicrobial agent that can kill micron-sized organisms rapidly but will not cause actual harm to much larger organisms like animals or humans as it cannot penetrate deeply into their living tissues. It is safe when used in low concentrations for short durations. Clinical trials must be undertaken to gain experience in the best dosages and protocols to eradicate antibiotic-resistant microorganisms from the body.

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Introduction

The five antibiotic-resistant bacteria we incorporated in this research study included *Escherichia coli, Methicillin-resistant Staphylococcus aureus, Klebsiella pneumonia, Acinetobacter baumannii, and Pseudomonas aeruginosa.*

The World Bank Group in 2017[1] warned that 2050 drug-resistant infections could cause global economic damage compared to the 2008 financial crisis. AMR also threatens the achievement of several of the United Nations' sustainable development goals, particularly the targets for good health and well-being.[2]

Hospital costs were estimated to be over €900 million in 2007.[3] Outpatient costs were 10 million, and the productivity loss from work was €150 million. Further, productivity loss due to mortality infection was estimated to be approximately €450 million annually. These economic costs will be considerably higher using the figures reported in the Global Burden of bacterial antimicrobial resistance in the 2019 report.[4]

Given the widespread nature of AMR bacteria worldwide and that antibiotics are becoming less and less effective, finding other more natural and novel ways of eradicating these killers is essential. Chlorine dioxide is a safe and effective way of eliminating these AMR bacteria without creating further resistance.

The discovery of new naturally derived antibacterial agents with new mechanisms of action remains a high priority globally.[5]

Post-treatment of Antibiotic-resistant bacteria

More than 150 antibiotics have been developed since the discovery of penicillin in 1940, and for most antibiotics available, resistance has emerged to the bacteria being treated.[6] On this basis, by 2050, the death toll could be staggering - one person every three seconds. Bacteria have resisted almost every antibiotic developed in the last 50 years.[4]

There is a positive correlation between the use and prevalence of antibiotics with higher rates in countries with higher use.[6] Inappropriate prescribing by doctors who incorrectly prescribe antibiotics for other infections, such as viral or fungal infections, rather than bacterial infections. There is a hypothesis that the excessive use of antibiotics is correlated with inappropriate prescription and administration of antibiotic therapy.[7] The irresponsible use of antibiotics is the core problem of antibiotic resistance.

The influence of antibiotics is now fading due to the progressive rise of resistance observed among all antimicrobial drugs.[8] Increased antibiotic resistance is driven by a combination of germs exposed to antibiotics and the spread of those germs and their mechanisms of resistance. Antibiotics are not only becoming less effective, but their use can also cause dysbiosis, especially in the intestines or in places of secondary infections. The effects on human health can be catastrophic: excessive reuse of antibiotics has been shown to destroy most of the natural intestinal flora.[9]

Pharmaceuticals are also unwilling to invest in developing antibiotics for several reasons, including low returns in the market, restrictions on antibiotic use, scientific difficulties in developing antibiotics, and the existing regulatory environment.[3] The route to find new antibiotics and develop them into drugs is long and expensive. It costs 800 million to 1 billion dollars to bring a new drug to market; on average, it takes over ten years to enter the clinic.[8] Due to the time pressure we face in the battle against AMR, a different approach to exploring alternatives to antibiotic therapy is needed.

Compared with all underlying causes of death in the Global Burden of Diseases, Injuries, and Risk Factors Study (GBD) 2019, antimicrobial resistance (AMR) would have been the third leading GBD Level 3 cause of death in 2019, based on the counterfactual of no infection; only ischaemic heart disease and stroke accounted for more deaths that year.[10] Moreover, many nosocomial pathogens may not be eliminated by the usual cleaning; thus, they can survive for extended periods in hospitals, indoor air, and surfaces and contribute to the transmission of infections.[11]

Alternative treatments represent a promising field of investigation. It is, therefore, imperative that new, novel treatments of AMRs are pursued, and this is the foundation of this research – using natural substances to eradicate AMRs such as MRSA, *E coli, S aureus, K pneumoniae, A baumannii*, and *P aeruginosa*, that do not create further resistance. The discovery of new naturally derived antibacterial agents with new mechanisms of action remains a high priority globally.[5]

Natural antimicrobials have been used successfully in treating bacteria[12] and have been the primary source of medicines throughout human existence, which one should not forget. Natural products, including medicinal plants, remain widely popular today, with approximately 80% of the world's population relying on herbal products and related supplements as part of their healthcare regimen.[13]

Natural products such as *Rosmarinus officinalis*[12] and *Mangifera indica L.*, Anacardiaceae,[14] antimicrobial peptides, plant essential oils and their combinations have proven to be quite effective in inhibiting a wide selection of bacterial pathogens, including the five AMR bacteria in this study.[15]

This research examines the eradication of antibiotic-resistant MRSA, *Escherichia coli*, *Klebsiella pneumonia*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa* with ampoules of CDSpure® containing 2,990 ppm per 5 ml ampoule[16] chlorine dioxide in vitro.

Importance of the five Antimicrobial-resistant bacteria

Acinetobacter Baumannii

Acinetobacter baumannii, comprises gram-negative, strictly aerobic, nonfermenting, non-fastidious, non-motile, catalase-positive, and oxidase-negative bacteria.[22,23]

Although *A. Baumannii* accounts for a relatively low percentage of overall bacteraemia cases, multi-drug resistance is globally problematic for this species.[24] Globally, over 71% are multi-drug resistant.[25] In the United States, 27% of mechanically ventilated patients were colonized with a multidrug-resistant strain of *A. baumannii*.[26] *A. baumannii* utilizes resistance strategies, including chromosomal β-lactamases, efflux pumps, and aminoglycoside-modifying enzymes. After human serum albumin exposure, *A. baumannii* upregulates the transcription of β-lactamases, indicating potentially inherent antimicrobial resistance mechanisms in serum.[27]

Due to the prevalence of infections and outbreaks caused by multi-drug resistant *A. baumannii*, few antibiotics are effective for treating conditions caused by this pathogen.[28]. Many reports have shown that *A. baumannii* rapidly develops resistance to antimicrobials, and multidrug-resistant strains have been isolated.[29] The World Health Organization (WHO) has also assigned *A. baumannii* as a critical priority pathogen posing a significant threat to human health, and towards which new antibiotics are urgently needed.[30]

Escherichia coli

E. coli frequently resist multiple classes of antimicrobials amongst strains that cause UTI and bacteraemia exceeding 50%.[32] *E. coli* is a major cause of diarrheal diseases, peritonitis, colitis, bacteraemia, infant mortality, and urinary tract infections worldwide, costing billions of dollars to treat and killing roughly 2 million humans annually.[33]

E. coli is a versatile Gram-negative bacterium, easily found and amenable to natural and random genetic alteration.[34] It is 1-3 x 0.4-0.7 μm in size and 0.6 to 0.7 μm in volume.[35]

Klebsiella pneumoniae*K. pneumoniae* strains are commonly classified as opportunistic, hypervirulent (hyKp), or multidrug-resistant (MDR).[36] While the classic *K. pneumoniae* (cKp) consists of opportunistic strains frequently associated with nosocomial infections, the hypervirulent strains are regarded as community-acquired bacteria that can infect people of all ages, including healthy individuals.[37,38] The rapid spread of multidrug-resistant *K. pneumoniae* strains is a major global health threat, as these strains are responsible for many hospital infections with high morbidity and mortality.

Klebsiella pneumonia, is described as a gram-negative, encapsulated, and non-motile bacterium.[39]

Methicillin-Resistant Staphylococcus Aureus (MRSA)

MRSA is considered one of the most dangerous nosocomial pathogens causing many hard-to-treat infections in hospitals and was named Hospital Associated MRSA (HA-MRSA).[40] Over the past 20–25 years, MRSA was isolated from community settings, and thus Community Associated MRSA (CA-MRSA) has emerged.[40]

MRSA can cause various organ-specific infections, the most common being the skin and subcutaneous tissues, followed by invasive infections like osteomyelitis, meningitis, pneumonia, lung abscess, and empyema. Infective endocarditis caused by MRSA is associated with increased morbidity and mortality compared to other organisms and is linked to intravenous drug abuse.[41]

Methicillin-resistant Staphylococcus aureus (MRSA) is a gram-positive coccus that is both catalase- and coagulase-positive.[42]

Pseudomonas aeruginosa

P. aeruginosa is among the five leading causes of nosocomial bacteremia, frequently leading to sepsis.[43]

P. aeruginosa is an important Gram-negative opportunistic pathogen that causes many severe acute and chronic infections with high morbidity and mortality rates as high as 40%. What makes *P. aeruginosa* a particularly challenging pathogen is its high intrinsic and acquired resistance to many of the available antibiotics.[44]

It is an opportunistic human pathogen capable of causing many life-threatening acute and chronic infections, particularly in patients with compromised immune defense. It is particularly important since it is the leading cause of morbidity and mortality in cystic fibrosis (CF) patients. It is one of the top nosocomial pathogens affecting hospitalized patients while intrinsically resistant to a wide range of antibiotics.[45]

Chlorine Dioxide (ClO_2) Antimicrobial

Chlorine dioxide (ClO_2) is a yellow to reddish-yellow gas that can decompose rapidly in the air.[48] Chlorine dioxide has a molar weight of 67.452

g/mol, and water solubility at sea level is 3.01 g/L (3000 ppm) at 25 °C and 34.5 mm Hg.[49,50,51]
It is an effective biocide at concentrations as low as 0.1 ppm and over a wide pH range. Even in cold water, it is ten times more soluble than chlorine.[52]
Chlorine dioxide has a lower oxidation potential than ozone and chlorine. The optimal pH is between pH 6.0 and pH 10.0 and is generally more effective against microorganisms at pH above 8.0 than chlorine.[53]
When it reacts in water, it forms chlorite ions, a very reactive chemical that can kill bacteria and microorganisms in any solution.
Chlorine dioxide rapidly kills bacteria, viruses, and *Giardia* and is effective against *Cryptosporidium*.[52] Chlorine dioxide also improves taste and odour, destroys sulphide and phenols, controls algae, and neutralizes iron and manganese ions. It is an effective biocide at concentrations as low as 0.1 ppm and over a wide pH range.

Chlorine Dioxide, Biofilms, and Resistance
Chlorine dioxide is more suitable for therapeutic use since it can penetrate and eliminate biofilm. According to Simpson et al. (1993)[54] chlorine dioxide can remove biofilms swiftly because it is highly soluble in water. Unlike ozone, it does not react with the extracellular polysaccharides of the biofilm. This way, chlorine dioxide can penetrate biofilms rapidly to reach and kill the microbes living within the film. Penetrating biofilms to eradicate microorganisms is a real challenge for both natural and allopathic medicine.
Biofilm is a three-dimensional structure formed by microbial cells that adhere to biotic or abiotic surfaces under various physiological and environmental factors that still need to be identified. Further, these cells continuously multiply and produce extracellular polymeric substances (EPS), forming a matrix encasing these microbes.
Biofilms are aggregates of microbial cells enveloped by self-produced exopolysaccharide matrices on biotic or abiotic surfaces. Biofilms demonstrate considerable protection against antibiotics, host immune defense, and adverse environmental conditions than free-living cells.[55] It is estimated that 65–80% of human infections are caused by biofilm-forming bacteria.[56]
Biofilms are estimated to be 1000 times more antibiotic-resistant than free-living cells.[57] The interplay between bacterial cells and environmental factors triggers biofilm formation. Chlorine dioxide can penetrate and eradicate biofilms, a significant advantage over many antibiotics that cannot do this.
Another very significant advantage of the therapeutic use of chlorine dioxide over antibiotics is that it cannot create antibiotic resistance in bacteria.[58] The presence of the four amino acids (cysteine, methionine, tyrosine, and tryptophan) and especially cysteine and biological thiols play a crucial role in all living systems, including microbes, so that no microbe can develop a resistance against chlorine dioxide.[59]
Chlorine dioxide penetrates bacterial cell walls and reacts with vital amino acids in the cell's cytoplasm to kill the organism. The by-product of this reaction is chlorite, which is not known to pose significant environmental or human health risks.

Safety and Efficacy of Chlorine Dioxide
In 1967, the US EPA first registered the liquid form of chlorine dioxide for use as a disinfectant and sanitizer.[49]
Chlorine dioxide is a size-selective antimicrobial agent that can kill micron-sized organisms rapidly but cannot harm much larger organisms like animals or humans as it cannot penetrate deeply into their living tissues.[59] Chlorine dioxide cannot penetrate deeply into the tissues of larger organisms, and the circulation of larger organisms provides a constant supply of antioxidants, offering protection against the effects of chlorine dioxide oxidants.[60]
Other research has shown that chlorine dioxide, a strong oxidant, can inhibit or destroy microorganisms at concentrations ranging from 1 to 100 ppm, producing potent antiviral activity, inactivating > or equal to 99.9% of the viruses within 15 seconds for sensitization.[61,62,63,64]
Georgiou (2021)[65] successfully showed the efficacy of chlorine dioxide against MRSA in-vitro, with growth inhibition of 99.99% -100% in even small concentrations.
Ongoing research at the Innerlight Biological Research Foundation has been investigating the clinical usage of chlorine dioxide, including nonspecific spores, bacteria, and viruses, for many applications for over twenty-five years.[66]
Extensive clinical applications of chlorine dioxide to Epstein-Barr virus (EBV), cytomegalovirus (CMV); hepatitis virus A; B; HIV (AIDS virus), and others are being used continually.[66]
On October 14, 2020, the BOLIVIA parliament passed a bill (ley 1351)[67] that allows chlorine dioxide to be used as a medicine against COVID-19. Chlorine dioxide has been used successfully in countries like Bolivia, Mexico, Peru, Brazil, and Colombia. In Bolivia, Law No. 1351 of 2020 (Official Gazette of Bolivia, 2020) was approved that authorized the preparation, commercialization, supply, and use of the chlorine dioxide solution for prevention and treatment in the face of the COVID-19 pandemic.

More recently, in 2021, a study by Insignares-Carrione[58] performed to determine the effectiveness of oral chlorine dioxide in treating COVID-19 showed that chlorine dioxide is effective in treating COVID-19.

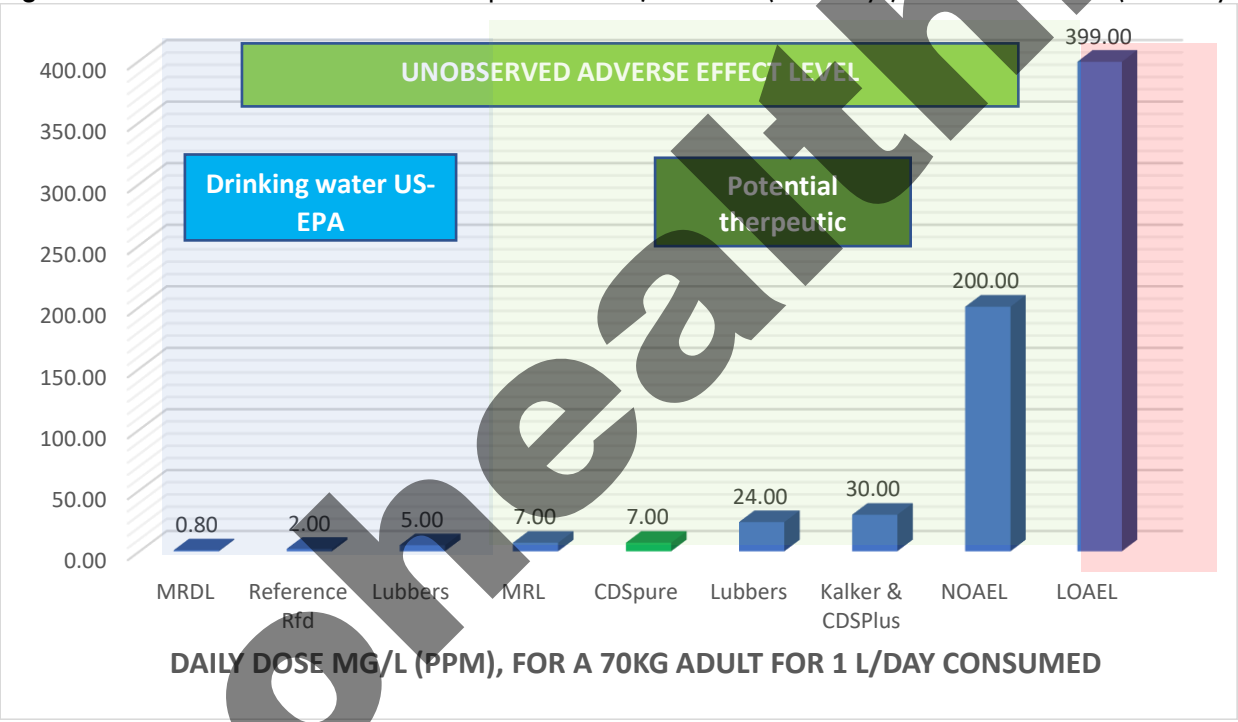
Chlorine dioxide has three atoms; scientists call this bond an "Unstable" or "Negatively Charged" ion.[69] When this bond of atoms separates, it creates a very tiny subatomic pulse of energy. This pulse happens when chlorine dioxide gets around pathogens in the body. It only attacks "acidic" and "anaerobic" microbes (viruses, bacteria, fungi, or parasites) in the body and will not harm the microbiome. These bacteria survive only without oxygen and include many pathogenic organisms.[66]

Anaerobic organisms have not developed adequate defenses against the onslaught of oxygen, particularly nascent oxygen, and quickly succumb to its lethal action. All the healthy cells that need oxygen to live or are "alkaline" are safe.[69]

Toxicology of Chlorine Dioxide

Figure 1 illustrates the reference levels for drinking water disinfection from the US Environmental Protection Agency (blue zone). The levels of potential therapeutic efficacy in test animals at zero toxicity are shown in the green zone. The toxic levels are shown in the red zone (above 399 ppm). The level of 5 ppm that eradicated 99% or more antibiotic-resistant bacteria in this study is within the blue/green area of zero toxicity.

Figure 1. Chlorine Dioxide Dose Used in Repeated-Dose, Subacute (<28 days) or Chronic Studies (>90 days)



Chlorine dioxide is added to drinking water to protect people from harmful bacteria and other microorganisms.[49] Most people are exposed to small amounts of chlorine dioxide and chlorite by drinking treated water, including food, as it is used as a disinfectant in the food industry.

There is no evidence that chlorine dioxide or chlorite affects reproduction in humans. Reproductive studies in male animals do not consistently demonstrate alterations in spermatogenic indices, abnormal morphology, or motility; however, reported effects appear at doses higher than the adverse developmental effects. Similarly, alterations in hematologic parameters occur at higher doses. No information was located regarding death in humans following oral exposure to chlorine dioxide.[49,69].

In a study by Scatina,[70] human volunteers drank chlorine dioxide in a solution of up to 24 ppm and showed no adverse effects.

Methodology

Materials and Methods

This study used five antibiotic-resistant bacteria: *E coli, S aureus, K pneumoniae, S pneumoniae, A baumannii*, and *P aeruginosa*.

All were obtained from a certified laboratory (ATTC, Germany)[71] in a frozen vial and grown on blood agar plates.

A sample of each bacteria was taken from the isolated cultures using a sterilized loop in a Safety

Class II cabinet from the culture plates and placed in sterile tubes with 5 ml of Tryptic Soy Broth (TSB). These culture tubes were incubated at 37 degrees Celsius in a Heraeus incubator. Once at a reasonable count, the tubes were frozen and then stored in liquid nitrogen at -176 degrees Celsius until use.

Counting Bacteria

When quantifying bacteria in laboratories, it is common to count colony-forming units, which is a simple method that gives a good general idea of cell viability. One major disadvantage is that it takes days to get results, which would differ from tech to tech based on sample preparation techniques and conditions.

This study obtained bacterial counts using the QUANTOM Tx™ Microbial Cell Counter from Logos Biosystems.[72] It is an image-based, automated cell counter that can identify and count individual bacterial counts 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 against dead cells.

To prepare the sample for the QUANTOM counter, 10 microlitres (µl) of the culture medium was taken using a DLAB electronic pipette previously calibrated and placed in a 1.5 ml sterilized Eppendorf tube. To this was added 2 µl of Viable Cell Staining Dye, which was incubated in a Heraeus incubator at 37 degrees centigrade for 30 minutes. To this sample, we added 8 µl of Buffer to enhance the fluorescent signal.

Preparing Chlorine Dioxide Solution

To prepare the chlorine dioxide, a 5 ml ampoule of Chlorine dioxide called CDS Pure® containing exactly 2,990 ppm chlorine dioxide (CAS 10049-04-41) manufactured by AQARIUS pro-life was used for the experiments.

This is a ready-to-use, sterilized chlorine dioxide solution in a sealed glass ampoule that is highly pure nano-filtered, pH neutral, and chlorine free. The chlorine dioxide solution (demineralized water, chlorine dioxide) contains no residues, silver ions, or nanoparticles.

Given that each 5 ml ampoule contains exactly 2,900 ppm of chlorine dioxide, it is easy to determine how many milliliters are required. The concentrations used varied between 1 – 7 ppm of chlorine dioxide. The amounts used for each concentration was 1 ppm = 1.667 µl CDS Pure®; 2 ppm = 3.344 µl; 3 ppm = 5.016 µl; 4 ppm = 6.688 µl; 5 ppm = 8.336 µl; 6 ppm = 10.032 µl and 7 ppm = 11.704 µl. The exposure time was irrelevant as the disinfection was immediate, within seconds.

To these tubes was added chlorine dioxide at different concentrations. The 5 ml CDS concentration of chlorine dioxide used in the experiment ranged from 1.667 µl (1 ppm) to 11.704 µl (7 ppm), added to the tubes with a DLAB electronic pipette and mixed gently for each bacteria.

A control tube was also prepared from the same culture medium for each experimental tube. According to the amount of chlorine dioxide applied to the experimental tube, the same quantity of distilled water was added to the control tube to keep the dilution factor constant.

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 8 mins 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 settings for the different bacteria were set according to the shape and size of the various bacteria to optimize the count.

Results

To evaluate the disinfection potential of chlorine dioxide on the five types of antibiotic-resistant bacteria in this study, 5 ml ampoules of CDSpure® were used throughout, as these ampoules contain a standardized 2,990 ppm concentration. The concentrations were from 1 ppm up to 7 ppm, in 1 ppm increments.

The control and experimental samples at the various concentrations were conducted in triplicate, for which an average was taken for each. These values were compared with the control sample without the CDSpure® chlorine dioxide.

Generally, with all five bacteria studies, there was a greater than 95% disinfection at the maximum concentration of 7 ppm chlorine dioxide, with some species being eradicated at lower concentrations of 4 – 7 ppm (t-test, $p<0.01$).

Let us examine each bacteria species individually in the tables and figures below.

Acinetobacter baumannii disinfection

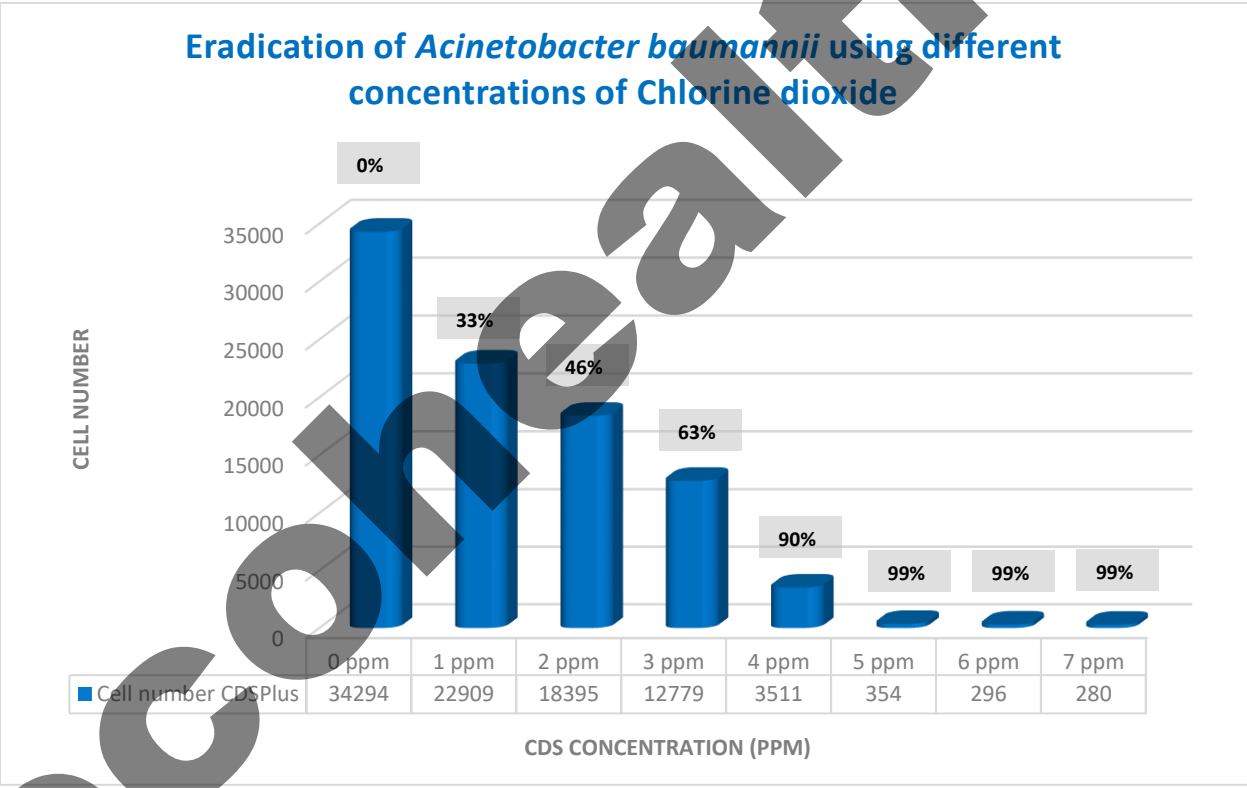
With *Acinetobacter baumannii*, there was a 99% disinfection at 5, 6 and 7 ppm chlorine dioxide concentrations (t-test, $p<0.01$) (Table 1, Figure 2).

Table 1: Comparison of Bacterial Counts of Acinetobacter baumannii Before and After Chlorine Dioxide Exposure.

CDS concentration (ppm)	CDS concentration (µl)	Cell concentration (cells per mL)		Size (µm)		Cell number		Difference in cell number	% difference in cell number
		C	E	C	E	C	E		
0 ppm	0	1.19E+09	1.19E+09	1.1	1.1	34293	34293	0	0
1 ppm	1.67	1.19E+09	7.95E+08	1.1	1.1	34293	22909	11384	33.20
2 ppm	3.34	1.19E+09	6.39E+08	1.1	1.1	34293	18395	15898	46.36
3 ppm	5.02	1.19E+09	4.44E+08	1.1	1.1	34293	12779	21514	62.74
4 ppm	6.69	1.19E+09	1.22E+08	1.1	0.9	34293	3511	30782	89.76
5 ppm	8.36	1.19E+09	1.23E+07	1.1	0.8	34293	353	33940	98.97
6 ppm	10.03	1.19E+09	1.03E+07	1.1	0.7	34293	295	33998	99.14
7 ppm	11.71	1.19E+09	4.21E+07	1.1	0.7	34293	279	34014	99.19

C = Control; E = Experimental

Figure 2. Comparison of Control vs. Experimental of Acinetobacter baumannii coli Using Chlorine Dioxide at Different Concentrations



Escherichia coli disinfection

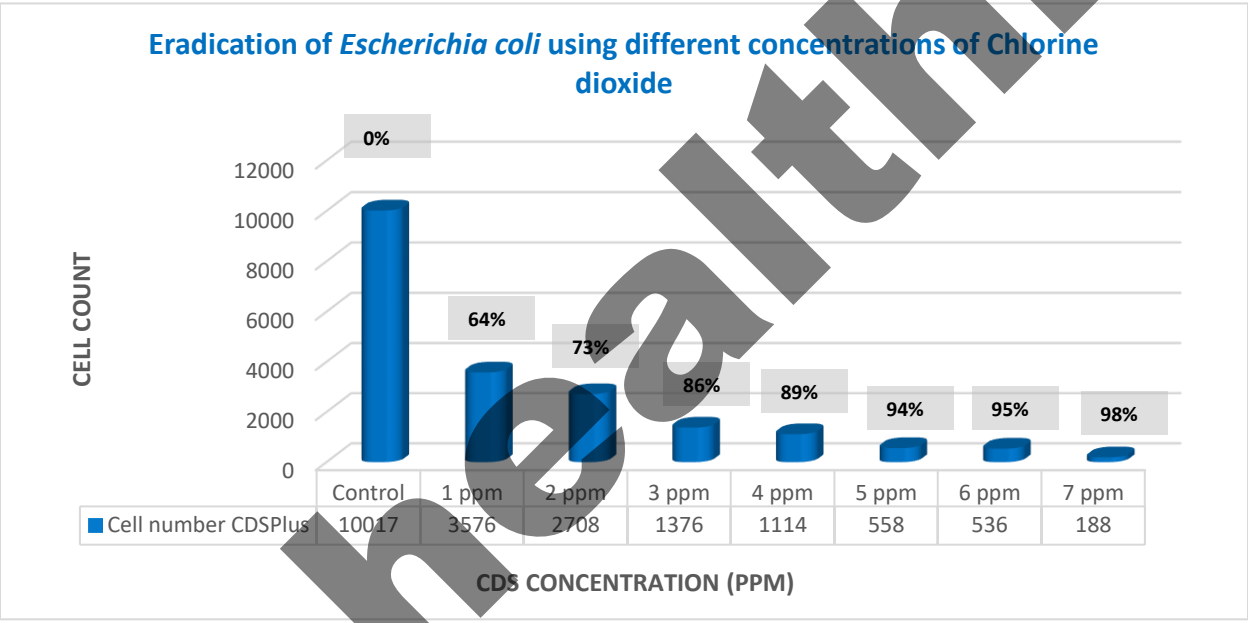
With the E. coli, there was a > 98% disinfection at a chlorine concentration of 7 ppm (t-test, $p<0.01$) (Table 2, Figure 3).

Table 2: Comparison of Bacterial Counts of Escherichia coli Before and After Chlorine Dioxide Exposure.

CDS concentration (ppm)	CDS concentration (µl)	Cell concentration (cells per mL)		Size (µm)		Cell number		Difference in cell number	% difference in cell number
		C	E	C	E	C	E		
0 ppm	0	4.16E+00	4.16E+00	1.8	1.8	10017	10017	0	0
1 ppm	1.67	4.16E+00	8.09E+07	1.8	1.8	10017	3576	6441	64.30
2 ppm	3.34	4.16E+00	6.16E+07	1.8	1.9	10017	2708	7309	72.96
3 ppm	5.02	4.16E+00	3.18E+07	1.8	1.9	10017	1376	8641	86.27
4 ppm	6.69	4.16E+00	2.16E+07	1.8	1.9	10017	1114	8903	88.88
5 ppm	8.36	4.16E+00	1.14E+07	1.8	1.9	10017	558	9459	94.43
6 ppm	10.03	4.16E+00	3.82E+06	1.8	1.9	10017	536	9481	94.65
7 ppm	11.71	4.16E+00	2.87E+05	1.8	1.8	10017	188	9829	98.12

C = Control; E = Experimental

Figure 3. Comparison of Control vs. Experimental of Escherichia coli Using Chlorine Dioxide at Different Concentrations



Klebsiella pneumoniae disinfection

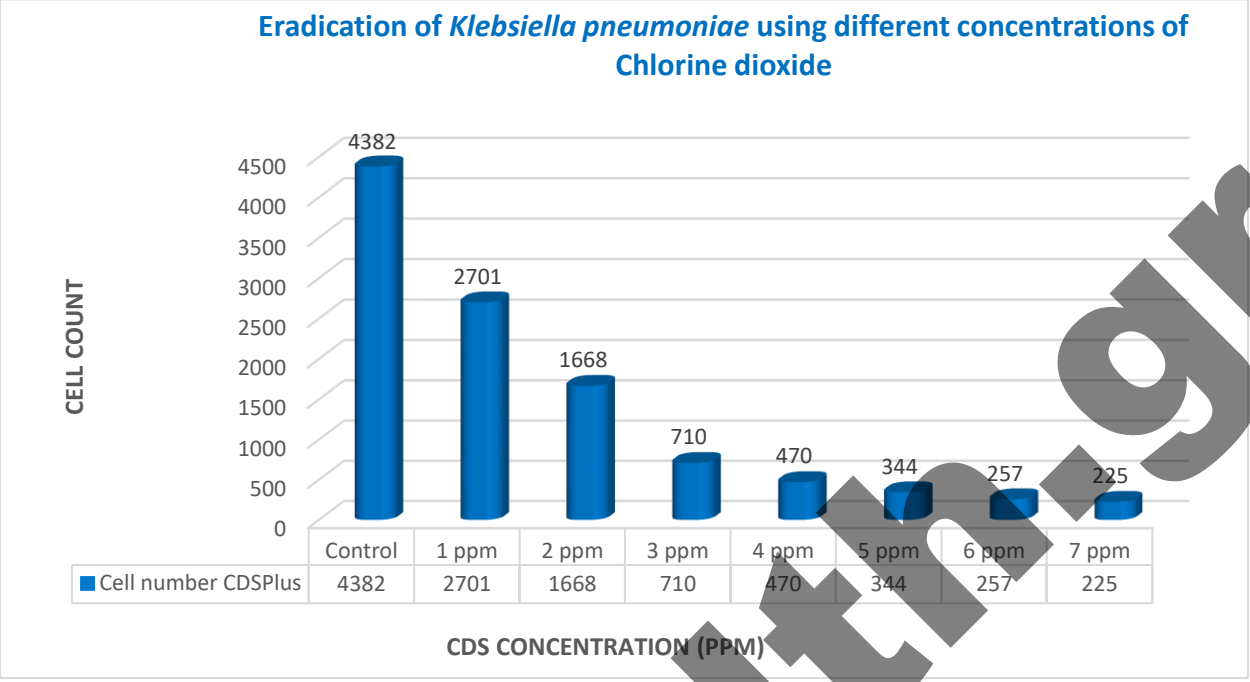
With the K. pneumoniae bacteria, there was a > 94% disinfection at a chlorine concentration of 6 and 7 ppm (t-test, $p<0.01$) (Table 3, Figure 4).

Table 3: Comparison of Bacterial Counts of Klebsiella pneumoniae Before and After Chlorine Dioxide Exposure.

CDS concentration (ppm)	CDS concentration (µl)	Cell concentration (cells per mL)		Size (µm)		Cell number		Difference in cell number	% difference in cell number
		C	E	C	E	C	E		
0 ppm	0	1.03E+08	1.03E+08	1.1	1.1	4382	4382	0	0
1 ppm	1.67	1.03E+08	6.25E+07	1.1	1.1	4382	2701	1681	38.36
2 ppm	3.34	1.03E+08	3.86E+07	1.1	1.2	4382	1668	2714	61.94
3 ppm	5.02	1.03E+08	1.64E+07	1.1	1.2	4382	710	3672	83.80
4 ppm	6.69	1.03E+08	1.06E+07	1.1	1.1	4382	470	3912	89.27
5 ppm	8.36	1.03E+08	6.72E+06	1.1	0.9	4382	344	4038	92.15
6 ppm	10.03	1.03E+08	5.97E+06	1.1	0.8	4382	257	4125	94.14
7 ppm	11.71	1.03E+08	3.82E+06	1.1	0.8	4382	225	4157	94.87

C = Control; E = Experimental

Figure 4. Comparison of Control vs. Experimental of Klebsiella pneumoniae Using Chlorine Dioxide at Different Concentrations



Methicillin Resistant Staphylococcus Aureus (MRSA) disinfection

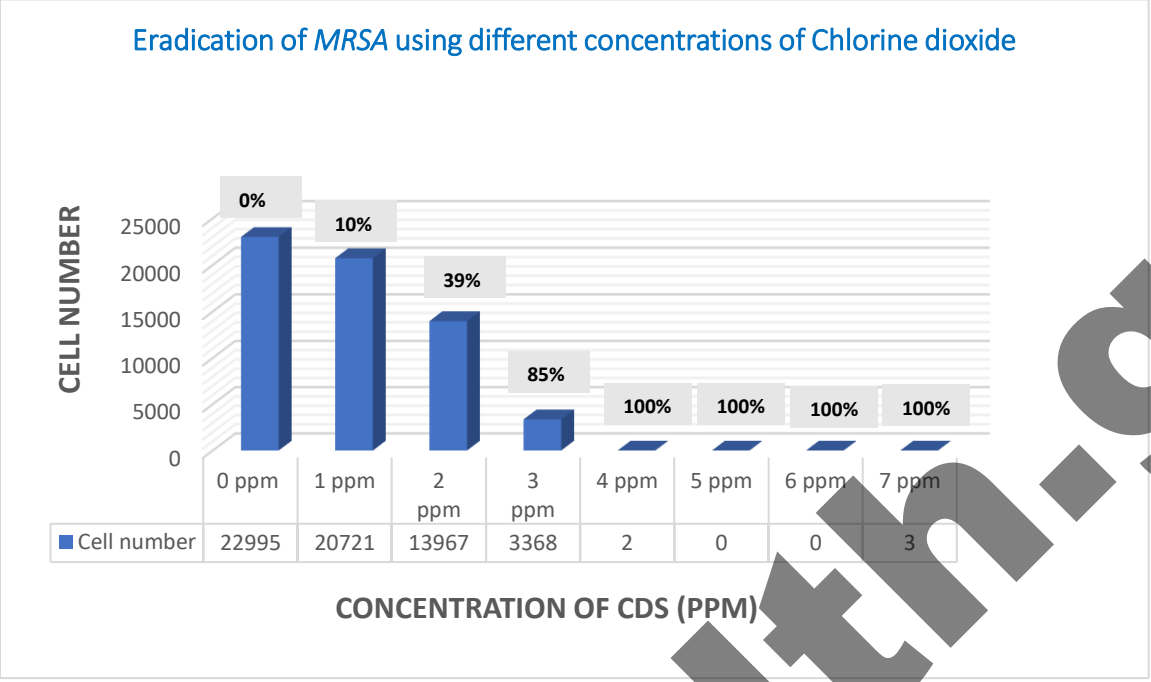
With the MRSA, there was a > 99% disinfection at a chlorine concentration of 4 - 7 ppm (t-test, $p<0.01$) (Table 4, Figure 5).

Table 4: Comparison of Bacterial Counts of MRSA Before and After Chlorine Dioxide Exposure

CDS concentration (ppm)	CDS concentration (µl)	Cell concentration (cells per mL)		Size (µm)		Cell number		Difference in cell number	% difference in cell number
		C	E	C	E	C	E		
0 ppm	0	5.32E+08	5.32E+08	2.6	2.6	22995	22995	0	0
1 ppm	1.67	5.32E+08	4.80E+08	2.6	2.1	22995	20721	2274	9.89
2 ppm	3.34	5.32E+08	3.24E+08	2.6	1.6	22995	13967	9028	39.26
3 ppm	5.02	5.32E+08	7.80E+07	2.6	1.1	22995	3368	19627	85.35
4 ppm	6.69	5.32E+08	3.47E+04	2.6	0.8	22995	2	22993	99.99
5 ppm	8.36	5.32E+08	0.00E+00	2.6	0.0	22995	0	22995	100.00
6 ppm	10.03	5.32E+08	0.00E+00	2.6	0.0	22995	0	22995	100.00
7 ppm	11.71	5.32E+08	6.95E+04	2.6	1.1	22995	3	22992	99.99

C = Control; E = Experimental

Figure 5. Comparison of Control vs. Experimental of MRSA Using Chlorine Dioxide at Different Concentrations



Pseudomonas aeruginosa disinfection

With the P. aeruginosa bacteria, there was a >95% disinfection at a chlorine concentration of 7 ppm (t-test, $p<0.01$) (Table 5, Figure 6). This research indicates that chlorine dioxide solution is an effective natural substance that can eradicate all the species of antibiotic-resistant bacteria tested. The concentrations of chlorine dioxide required to achieve a 95% or greater disinfection for the five antibiotic-resistant bacteria is from 4 - 7 ppm, which are within potentially safe limits a (see Figure 1).

Figure 6. Comparison of Control vs. Experimental of Pseudomonas aeruginosa Using Chlorine Dioxide at Different Concentrations

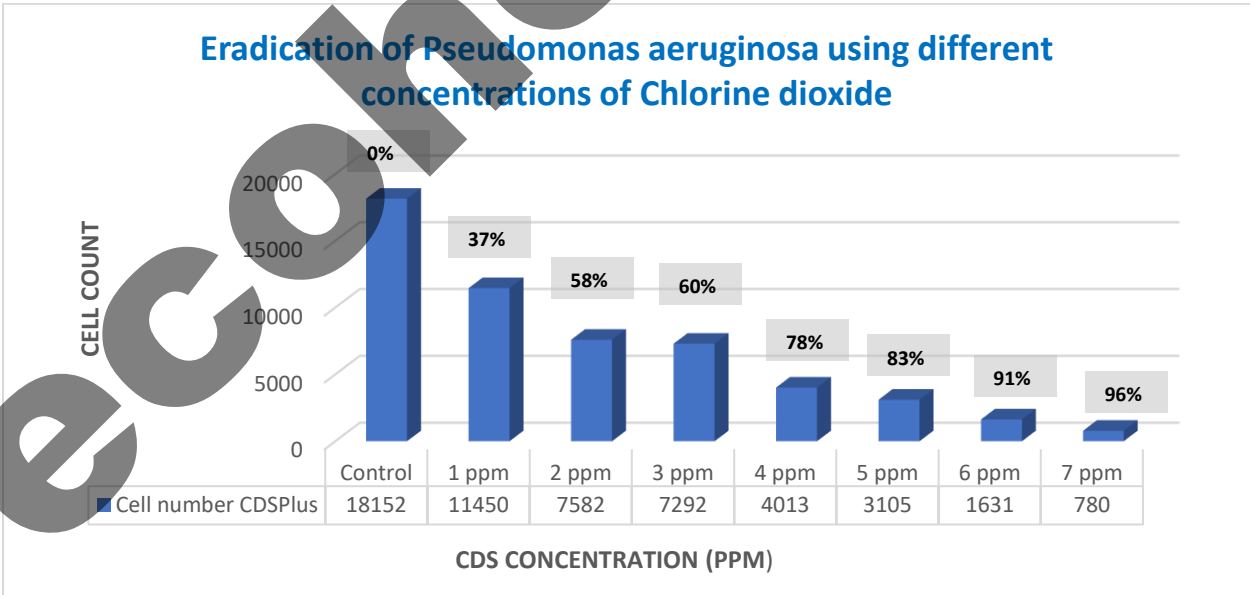


Table 5: Comparison of Bacterial Counts of Pseudomonas aeruginosa Before and After Chlorine Dioxide Exposure.

CDS concentration (ppm)	CDS concentration (µl)	Cell concentration (cells per mL)		Size (µm)		Cell number		Difference in cell number	% difference in cell number
		C	E	C	E	C	E		
0 ppm	0	4.26E+08	4.26E+08	2.5	2.5	18152	18152	0	0
1 ppm	1.67	4.26E+08	2.66E+08	2.5	2.5	18152	11450	6702	36.92
2 ppm	3.34	4.26E+08	1.76E+08	2.5	2.6	18152	7582	10570	58.23
3 ppm	5.02	4.26E+08	1.60E+08	2.5	2.5	18152	7292	10860	59.83
4 ppm	6.69	4.26E+08	8.67E+07	2.5	2.5	18152	4013	14139	77.89
5 ppm	8.36	4.26E+08	5.28E+07	2.5	2.3	18152	3105	15047	82.89
6 ppm	10.03	4.26E+08	3.68E+07	2.5	2.2	18152	1631	16521	91.01
7 ppm	11.71	4.26E+08	1.80E+07	2.5	2.4	18152	780	17372	95.70

C = Control; E = Experimental

Discussion

The rise of antibiotic-resistant bacteria poses a significant threat to public health worldwide. These resilient bacteria have developed resistance to conventional antibiotics, making infections increasingly challenging to treat and leading to a rise in mortality rates and higher healthcare costs. The reported annual mortality toll is projected to surpass 10 million by 2050 from antibiotic-resistant diseases, outnumbering cancer deaths.[1,2,3,4] This poses a grave threat to global health, as once quickly managed infections can now result in prolonged illnesses, leading to much suffering and a burden on the healthcare system.

The impact of research into alternative approaches for eradicating antibiotic-resistant bacteria cannot be understated. This research can save lives on a global scale through the following:

1. Improved Patient Outcomes: Alternative strategies can effectively treat infections once deemed untreatable. By successfully targeting antibiotic-resistant bacteria, these approaches can improve patient outcomes, reduce morbidity and mortality rates, and restore hope to individuals afflicted by resistant infections.
2. Reduced Healthcare Burden: The growing burden of antibiotic-resistant infections strains healthcare systems worldwide. By finding alternative ways to combat resistance, the load on healthcare resources can be alleviated, resulting in improved efficiency, reduced hospital stays, and decreased healthcare costs.
3. Prevention of Epidemics: If unchecked, resistant bacteria can cause widespread epidemics. Research on alternative approaches offers the opportunity to prevent the emergence and spread of resistant strains, ultimately safeguarding public health, and averting large-scale outbreaks.
4. Diversification of Treatment Options: Novel and natural treatments provide alternative approaches to combating antibiotic resistance. They expand the range of treatment options available to healthcare providers, ensuring they have multiple tools to fight resistant infections. This diversification increases the chances of successful treatment outcomes and helps address the limitations of conventional antibiotics.
5. Overcoming Resistance Mechanisms: Antibiotic-resistant bacteria have developed sophisticated mechanisms to evade the effects of traditional antibiotics. Novel and natural treatments offer the potential to target bacteria through different pathways, bypassing or overcoming existing resistance mechanisms, providing a fresh approach to combatting resistant strains, and reducing the likelihood of treatment failure.
6. Reduced Side Effects and Toxicity: Many conventional antibiotics have associated side effects and can be toxic to the body, primarily when used over extended periods. Natural treatments, like chlorine dioxide, may offer lower toxicity profiles and reduced side effects. This is particularly advantageous for vulnerable populations, such as children, pregnant women, and the elderly, who may be more susceptible to the adverse effects of conventional antibiotics.
7. Preservation of Gut Microbiota:[9] Conventional antibiotics often disrupt the balance of beneficial bacteria in the gut, leading to dysbiosis and potential long-term health consequences. Novel and natural treatments may offer targeted approaches that selectively eliminate harmful bacteria while preserving the diversity and function of the gut microbiota. This preservation is crucial as a healthy gut microbiome plays a vital role in immune function, digestion, and overall well-being.

8. Sustainable and Environmentally Friendly Solutions: The production and use of conventional antibiotics can have adverse environmental effects, including developing antibiotic-resistant strains in the environment. Natural treatments often come from renewable sources, such as plants or microbial-derived compounds, which can be produced sustainably. Additionally, these treatments may have minimal environmental impact as they often break down more readily, reducing the risk of long-term ecological damage.
9. Potential for Combination Therapies: Natural treatments can be combined with conventional antibiotics or other alternative therapies to create synergistic effects and enhance treatment outcomes. This approach allows for personalized and tailored treatment regimens, optimizing the potential benefits of each treatment modality. Combination therapies can be more effective in combating resistant bacteria, reducing treatment time, and preventing further development of resistance.
10. Accessible and Affordable Treatment Options: Access to conventional antibiotics can be limited or cost prohibitive in many parts of the world. Natural treatments derived from locally available resources may offer more accessible and affordable options for treating resistant infections. This can significantly impact global health, especially in resource-constrained settings where the burden of antibiotic resistance is high.

In the search for effective alternatives, chlorine dioxide has emerged as a promising solution for eradicating antibiotic-resistant bacteria. It works by disrupting the cellular structures of bacteria, preventing them from multiplying and causing further harm.

Advantages of Chlorine Dioxide

Chlorine dioxide has emerged as a promising weapon in the fight against these resilient pathogens. Its broad-spectrum activity, residual effectiveness, reduced likelihood of resistance, and environmental safety make it an invaluable tool in eradicating antibiotic-resistant bacteria. By embracing chlorine dioxide as part of comprehensive infection control strategies, we can significantly reduce mortality rates associated with these infections, safeguard public health, and address the growing problem of antibiotic resistance.

There are many advantages to using chlorine dioxide in the treatment of antibiotic-resistant microorganisms, such as:

Spectrum Activity: Chlorine dioxide has shown efficacy against many bacteria, including MRSA and other antibiotic-resistant strains. Its ability to target the bacterial cell wall and disrupt essential metabolic processes sets it apart as an effective antimicrobial agent.

Residual Effectiveness: Unlike traditional disinfectants, chlorine dioxide exhibits a residual effect, which protects surfaces even after initial application. This residual activity is crucial in healthcare settings where surfaces can become contaminated quickly, providing a tenacious defense against bacterial colonization and transmission.

Reduced Likelihood of Resistance Development: Chlorine dioxide's mechanism of action reduces the likelihood of bacteria developing resistance. Unlike traditional antibiotics that target specific bacterial components, chlorine dioxide attacks multiple cellular structures simultaneously, making it harder for bacteria to develop resistance mechanisms.

Eradication of Biofilms: Research studies have shown Chlorine dioxide to penetrate and eliminate biofilms. Biofilms are estimated to be 1000 times more antibiotic-resistant than free-living cells.[57]

Environmental Safety: Chlorine dioxide is recognized for its favourable ecological profile. It decomposes into harmless byproducts, leaving no toxic residues. This characteristic ensures that its use for disinfection purposes does not contribute to long-term environmental damage.

The Potential Impact: The importance of chlorine dioxide in eradicating antibiotic-resistant bacteria cannot be overstated. By providing an alternative treatment option, chlorine dioxide can help combat the growing problem of antibiotic resistance, reduce mortality rates, and alleviate the burden on healthcare systems. Its effectiveness against MRSA and other resistant strains can improve patient outcomes, shorter hospital stays, and reduce healthcare costs.

Cost-Effectiveness: The chlorine dioxide solution is cheap and can significantly reduce a country's healthcare costs over time.

Conclusions

This research has focused on studying the effectiveness of chlorine dioxide in vitro for some of the most critical antibiotic-resistant bacteria causing millions of deaths yearly on a global basis.

We have shown that the optimal concentration of chlorine dioxide that results in near complete disinfection in vitro is 7 ppm ($p<0.01$). This is a safe dosage to take as it is about the dosage used for water treatments.

What has been observed in vitro will most probably be seen in the human body. Like blood, chlorine dioxide releases oxygen when it encounters acidity, either from lactic acid or the microorganism's acidity.[58] When chlorine dioxide dissociates, it

releases oxygen into the blood, as do erythrocytes (red blood cells), through the same principle (known as the Bohr Effect), which is to be selective for acidity.

Chlorine dioxide is a size-selective antimicrobial agent that can kill micron-sized organisms rapidly but cannot cause actual harm to much larger organisms like animals or humans as it cannot penetrate deeply into their living tissues.[73]

There has been a lot of controversy around the use of chlorine dioxide. However, one must bear in mind that, like with any medication or supplement, the effects of exposure to any substance depend on the route of administration (e.g., inhaled, topical, or oral), the state of the molecule (i.e., gaseous, or aqueous), dose concentration, duration of exposure, personal traits and habits, and whether other chemicals or impurities are present.

Emphasizing that many toxicological studies have been carried out throughout the years with chlorine dioxide in humans and animals, showing its safety and efficacy as it has been used in many applications to ensure that humans are exposed to safe levels.

When used appropriately in the low doses required to neutralize microbes' chlorine dioxide has been proven safe. There were positive results in recent clinical trials with chlorine dioxide carried out for COVID-19 by Insignares-Carrione in 2021[58] where ultra-pure CDS chlorine dioxide (2,990 ppm) was administered intravenously to patients.

Moreover, Governmental approvals of chlorine dioxide solutions for the prevention and treatment of the COVID-19 pandemic in Bolivia became law in 2020.[75] By which an ethics committee was legally constituted and endorsed by the Bolivian Ministry of Health, which, through their clinical, scientific, and ethical research committees, is conducting its research on chlorine dioxide for use in different applications.

Further, extensive clinical applications of chlorine dioxide to Epstein-Barr virus (EBV), cytomegalovirus (CMV); hepatitis virus A; B; HIV (AIDS virus), and others are being used continually. Further research has been investigating the clinical usage of chlorine dioxide, including nonspecific spores, bacteria, and viruses over the years.[66]

There are hundreds more testimonials of the use of chlorine dioxide by volunteers that have been collected over the years by Jim Humble for various applications of chlorine dioxide.[76]

Clinical trials need to be conducted to gain clinical experience in what would work best in clinical practice. Clinicians using IV infusions can use the 5 ml CDSpure® (2,990 ppm) ampoules directly or orally when diluted to the required levels.

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