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RESEARCH ARTICLE

Eradication of Borrelia Burgdoferi *in vitro* using Chlorine Dioxide: A Novel Approach

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ABSTRACT

Lyme disease, which is caused by the spirochete *Borrelia burgdorferi* is the most prevalent tick-borne illness in the world today and has grown into a major public health problem during the last decade, irrelevant to decades of efforts from various health professionals.

The conventional treatment for Lyme disease is the use of a variety of antibiotics, but relapse often occurs when antibiotics are discontinued. There are several reasons why this relapse may occur, given that B. burgdorferi is a pleomorphic microorganism that can convert from vegetative spirochetes to a variety of different round bodies and biofilm colonies. Therefore, there is an urgent need for novel approaches that can eliminate all these different morphologies.

This has challenged many health practitioners around the world, not to mention the suffering of many afflicted people. In this study, Chlorine dioxide (CD) at different concentrations was tested for its effectiveness in vitro against B. burgdorferi using combined fluorescent and darkfield microscopy with Live-or-Dye staining methods. Our experiments demonstrated that it is possible to completely eradicate all forms of B. burgdorferi at specific concentrations of Chlorine dioxide. Our extensive research has shown that Chlorine dioxide can be used for the eradication of B. burgdorferi morphologies.

At certain concentrations of chlorine dioxide above 2 ppm the Borrelia morphologies appear to be eradicated as there is no motility of either spirochetes or round bodies, only biofilms visible. However, incubating again for another 7 days resulted in the Borrelia being motile again as they come out of the biofilms.

We, therefore, decided to conduct each experiment with what we termed the Regrowth Kill Test (RKT) by incubating the initial sample with the CD in an incubator at 37 degrees centigrade in the Campypack for 7-days. It appeared that the biofilms that are quickly formed as soon as the CD is added in the initial experiment, broke up and released small spirochetes and round bodies of different morphologies.

After numerous RKT experiments, it was determined that the concentration that resulted in a near complete disinfection of the spirochetes as well as round bodies was 30 ppm CD.

Introduction

Lyme disease (LD), also known as Lyme borreliosis, is caused by the different genospecies of the spirochaete bacterium Borrelia burgdorferi sensu lato group[1-3] and is the most reported tick-borne infection in Europe and North America, which is also endemic in many areas in Asia[3,4]

Borrelia consist of thin, spiral-shaped motile bacteria, with 7 to 11 flagellae in the periplasmic space, enveloped around the cell and attached to the poles. The genetic structure of Borrelia burgdorferi is quite unusual and the entire genome has been mapped and consists of a 950 Kbp linear chromosome and an additional 500,000 base pairs spread over 9 small linear and 12 circular plasmids.[88]

According to insurance claims data from 2010 – 2018, the CDC recently estimated ~476,000 diagnosed cases of LD every year in the USA.[5] As the most prevalent vector-borne disease in the Northern Hemisphere[6,7], it is now considered an escalating health threat that is costing well over $1 billion per year in the USA alone.[8]

The general medical treatment for LD is the use of a cocktail of antibiotics for a 2–4-week period or longer. However, studies have shown that 36-63% of patients treated with these antibiotics still suffer from persistent symptoms,[9] a condition that has been termed Post Treatment Lyme Disease (PTLD), characterized by infection-induced immune dysfunction or auto-immunity, chronic inflammation, persistent bacterial infections, neurological symptoms, and other tick-borne symptoms.

The purpose of this in vitro research is to find the correct dosage of a natural antimicrobial, chlorine dioxide, that can eradicate Borrelia and its pleomorphic morphologies and biofilms.

Post Treatment Lyme Disease (PTLD)

PTLD patients are generally treated with more antibiotics over a prolonged period, but this approach may be both dangerous[10,11] and ineffective.[12,13,14,15] The general conclusion from these studies was that additional antibiotics for PTLD did not benefit patients compared to a placebo group.

Pleomorphic Characteristics of Borrelia

The question of what causes PTLD is a long-standing one. One feasible explanation has to do with the fact that B. burgdorferi sensu lato is pleomorphic[16,17,18] as it can change its morphology from spirochaetes to round bodies that consist of cell wall deficient (CWD) forms, L-forms, or spheroplasts.[19,20]

Changes in the internal milieu with prolonged antibiotic use[21] can interfere with the cell wall synthesis leading to a considerable loss of peptidoglycan cell wall, leading to the morphological changes mentioned here. Additionally, various bacteria can also aggregate into biofilms.[22,23,24,25]

The in vitro pleomorphic or polymorphic characteristics of B. burgdorferi have been identified in several studies,[26,27,28,29,30,31] as well as in clinical samples.[22,32,33]

The flat-wave forms of B. burgdorferi spirochetes are the most common, but there are many other morphologies such as blebs, round bodies (RB) and cell wall deficient forms, looped or ring-shaped forms, and spirochete colonies or biofilm aggregates.[57, 58-72]

Hostile environments such as pH, temperature, oxidative stress, change of media composition, acidity/alkalinity, concentrations of salts, sugars, or other organic compounds or antibiotic treatments can all change the morphology from spirochetes to RB such as granules, cysts, spheroplasts or vesicles.[73-81]

Many researchers have mistaken the transformation of the motile forms into a RB as a decrease in pathogen viability. However, even though RBs are less motile than spirochetes, they are still able to revert to the active growing reproductive spirochetes when the conditions are favourable.[64, 70-73,74-76,80,82]

Antibiotic treatment of B. burgdorferi results in biphasic killing[83-84] – certain bacteria are eliminated but concomitantly another group of drug-tolerant cells appears known as persisters.[85-86] As the concentration of antibiotics reaches a certain threshold, only persister cells survive.[87] Persisters have not been genetically modified to develop resistance, they are simply tolerant to the antibiotics in a non-inheritable way.[88] Persisters can also transform to "reverters" (replicating forms) under the right conditions, and will therefore cause further relapses and chronic infection.[84-87]

Given the protective mechanisms involved in the pleomorphic abilities of B. burgdorferi,[34,35,36,37] this in vitro research is oriented to finding effective natural substances that can eradicate all pleomorphic forms, including the persisters and reverters.

Chlorine Dioxide (ClO_2)

This research chose Chlorine dioxide (ClO_2) for a variety of reasons. ClO_2 is a small molecule (0.214 nm) with a molecular weight of 67.46, and it forms a stable radical.[89] It is smaller than the

smallest virus, so can penetrate all areas of the body including the blood brain barrier.

ClO2 is an oxidizer, which is reduced to chlorite ion (ClO_2 -) by capturing an electron (ClO_2 + e- → ClO_2 -). It does no harm to the human microbiome.[90,91] as the redox potential (E°) is relatively high at 0.95V.

Another reason to choose ClO_2 was its ability to remove biofilms swiftly because it is highly soluble in water and can penetrate biofilms rapidly to reach and kill the microbes living within the film.[39 – 48]

Most research on Borrelia species has been focused mainly on antibiotic efficacy, the different pleomorphic forms of the organism, survival in multiple host types based on protein regulation, and persistent infections.[49] However, very little research has been published on Borrelia's susceptibility to disinfection using natural products such as chlorine dioxide.

Chlorine dioxide is bactericidal, viricidal, sporicidal, cysticidal, algicidal, and fungicidal.[92]

Several researchers in different research studies have shown that chlorine dioxide is a strong oxidant that can greatly inhibit and eradicate microorganisms at different concentrations ranging from 1 to 100 ppm. Indeed, it was shown that chlorine dioxide has potent antiviral activity, inactivating 99.99% of viruses upon a 15-second exposure.[92-96]

One of the remarkable properties of chlorine dioxide that has not been achieved by either allopathic or natural medicine, is the breakdown of protective biofilms, due to their hydrophilic properties. Unlike ozone, chlorine dioxide does not react with the extracellular polysaccharides of the biofilm, therefore it is capable of penetrating biofilms rapidly to reach and kill the microbes living within the film.

There are many reports that ClO_2 has virucidal activity.[98-102] The inactivation concentration against various viruses is 1-2 ppm in poliovirus,[98,99] 2.19 ppm in coronavirus which causes SARS.[100] 7.5 ppm in hepatitis A virus,[101] and 0.2 ppm in rotavirus.[102]

Safety of Chlorine Dioxide

Chlorine dioxide has been used for over 50 years and has been shown to be non-toxic and safe, with no adverse effects on health.

Chlorine dioxide has been used in areas such as water treatment, textile industries, bleaching of pulp and paper, and the elimination of unwanted tastes and odours.

Various toxicological tests have been tried over decades, such as drinking chlorine dioxide dissolved in water, injecting it directly into the blood and under the skin of animals and into the brains of mice, adding it to tissue cultures, as well as using it with burns, disinfecting seeds and insect eggs and injecting into the stalks of plants.[103,104]

One study with human volunteers had shown that drinking chlorine dioxide up to 24 ppm in water, demonstrated no adverse effects.[105]

Moreover, there was no evidence found of foetal malformations or birth defects with chlorine dioxide concentrations of up to 100 ppm in reproductive toxicology and teratology.[106-108]

Further studies with rats fed up to 1,000 mg/l for 6 consecutive months did not show any significant haematological effects. Feeding the rats for 9 consecutive months resulted in a decrease in red blood cell counts, haematocrit, and haemoglobin.

In other toxicity studies, rats[109] and honeybees[110] were fed chlorine dioxide in high doses of up to 100 ppm for two years, without showing any adverse effects.

Methodology

Materials and Methods

Types of Chlorine dioxide Used in Testing

The Chlorine dioxide (ClO_2) used in these experiments came in three different forms: CDSplus®[38] which is a patented, pure, buffered solution of chlorine dioxide solution (approx. 2.9g/l = 0.29%/l = 2,900 ppm) at a pH of 7. The other form was CDSPure® which is a chlorine dioxide solution in a sealed glass ampoule that has been nano-filtered and is pH neutral and chlorine free.[38] The third type is CDS which is a pure chlorine dioxide gas bubbled into distilled water.

Borrelia burgderfori bacteria were obtained from ATTC, Germany[50] in a frozen vial, with strain designation B31, which was thawed at room temperature, and aseptically transferred to a 10ml tube of fresh Barbour-Stonner-Kelly (BSK-H) growth media with 6% rabbit serum. One-tenth of this cell suspension was transferred to 2 additional 10ml tubes of fresh growth media. The remaining contents were transferred in cryovials and stored in liquid nitrogen.

The Borrelia was cultured in a specialized media Barbour-Stonner-Kelly (BSK-H) medium that is highly enriched with bovine serum albumin and contains 6% heat-inactivated rabbit serum which is critical for proper growth.[52]

The culture was incubated in a microaerophilic environment using a Campypack

placed in an ordinary incubator at the optimal temperature of 37° C.[52] Generally, spirochetes grow slowly and Borrelia burgdorferi doubles every 12 hours approximately in the correct anaerobic conditions provided by the Campypack as opposed to a 5% CO_2 incubator.

Specimens of Borrelia burgdorferi cultures or cells were examined on a slide with a coverslip using a Zeiss Axioplan 2 Imaging microscope equipped with a Zeiss darkfield Ultra condenser 1.2/1.4 (0.75-1.0) and a Zeiss darkfield oil immersion 40X objective lens. The microscope is also equipped with fluorescence capabilities, as well as a Zeiss Axiocam MRc5 colour camera.

The Borrelia spirochaete is thin and difficult to view using brightfield or phase contrast. Darkfield microscopy will allow the different morphologies to appear as bright objects surrounded by a dark background as they are stained by light, and the contrast allows for simple spirochete identification.51

In these experiments, fluorescence microscopy with darkfield was used simultaneously (Diascopy) as the darkfield condenser directs the excitation light with a strong inclination towards the sample, so very little light enters the objective. The image is intensely fluorescent on a dark background as all light is blocked by the spread of the excitation barrier filter.[56]

Live/Dead Differentiation of Lyme Spirochetes

Borrelia spirochetes cannot be counted on automatic microbial counters due to their size and pleomorphic characteristics. We, therefore, needed to devise a method to differentiate live from dead Borrelia forms and count them under the microscope.

To differentiate live and dead Borrelia forms and obtain the percent of live/dead cell measures, darkfield microscopy was used, along with fluorescent microscopy using a FITC filter cube (blue) and the Live-or-Dye™ Fixable Viability Staining Kits (488/515) from Biotium[53] which is designed for discrimination between live and dead cells during flow cytometry or microscopy.

Live-or-Dye™ Fixable Viability Stains are cell membrane impermeant amine-reactive dyes. The dyes can enter dead cells that have compromised membrane integrity and covalently label-free amines on intracellular proteins, so only the dead cells will fluoresce a bright, light-blue colour, with the live cells being far dimmer.[54]

Samples were prepared from the cryovials by adding 1µl of Live-or-Dye™ Fixable Viability Stain and adding it to 100µl of culturing broth (BSK-medium ready to use), to prepare the working stock of the day. From each working stock, 20µl were then added to 100µl of Borrelia culture, to produce the experimental tube, followed by incubation at room temperature for 30 minutes.

Once the incubation was complete, the Chlorine Dioxide (CD) solution was added to the experimental tube, depending on the number of experiments to be run on that day. Different CD concentrations were used depending on the experiment being conducted.

Immediately after CD was added to the experimental tube, a sample was taken and placed onto a slide with a coverslip and observed under darkfield/fluorescence microscopy (Dioscopy). The motility of the spirochaetes and round bodies was observed, and the percentage live/dead was estimated based on the fluorescence resulting from the Live-or-Dye™ Fixable Viability Stain.

The Regrowth Kill Test (RKT)

Preliminary experiments had found that the CD acts on the Borrelia immediately, making all the morphological forms immotile within seconds, so measuring contact times was superfluous.

However, if the immotile sample was left for a day or so in the incubator, and re-examined on the microscope, the small round bodies would regain their motility again.

It was therefore decided to add what we termed the Regrowth Kill Test, testing the sample with the added CD again 7-days after the initial viewing.

So, after exposure to the CD, the Borrelia cells were washed, and new BSK-H media added (500 ul) and placed back in the Campypack jar in a normal incubator at 37°C for a further 7-days to determine whether there was any further regrowth detectable over time.

One-week post-inoculation with the CD, the cells were again removed and re-examined under the darkfield microscope using the FITC fluorescent filter for spirochaete mobility. These experiments were run in triplicate and the average was taken.

The Borrelia was observed for motility of spirochetes and round bodies, as well as the fluorescence of the dead cells. Estimated counts were made of each for each concentration of CD used.

The ASTM E2315-16 standard test method was adapted and used in this study for assessing bactericidal activity in liquid suspension.[55] In addition, live/dead viability assessment of the samples via Biotium's fluorescent viability dye named Live-or-Dye™ viability stain. All disinfection efficacy tests were conducted in triplicate.

Results

Assessing Antimicrobial Sensitivity

To evaluate in vitro chemical antimicrobial sensitivity of B. burgdorferi spirochete and round body morphological forms, B. burgdorferi (B31-35210) was subjected to contact with the CD at different concentrations from 1 - 30 ppm, in 5 ppm intervals.

Mobility and viability of spirochetes and round bodies were evaluated using direct cell counting of live and dead cells – dead cells fluoresce a bright-blue colour using the FITC cube (excitation 450-490nm) – as well as motility assessment using darkfield microscopy.

All experiments were conducted in triplicate and compared with a control sample without disinfectant.

Microscopy images were taken, as well as video to determine the mobility of the Borrelia spirochaetes and round bodies.

Here are a few examples of the images obtained. The initial experiments found that any concentration of CD at 2 ppm or higher would result in the mobility of spirochaetes and round bodies being 0%, with large biofilms forming, whether this was viewed in darkfield or darkfield-fluorescence.

Figure 1A shows the control sample of Borrelia with no CD added – the image is teeming with live, motile spirochetes and round bodies of different morphologies. We have considered the control sample to have 100% motility.

Figure 1B is the same sample but seen under Fluorescent-Darkfield microscopy (Dioscopy) using an excitation wavelength of 450-490nm with the FITC cube. Only the dead bacteria will phosphoresce a bright, light-blue colour using the Live-or-Dye™ viability stain. In this case, as all the bacteria are alive, there is very little phosphorescence seen. We can refer to this as 0% phosphorescence, which is equivalent to 100% motility or live.

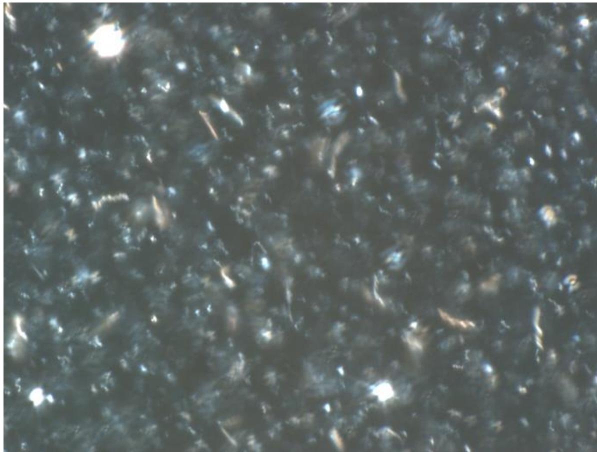


Figure 1A - Control under Darkfield (no CD)

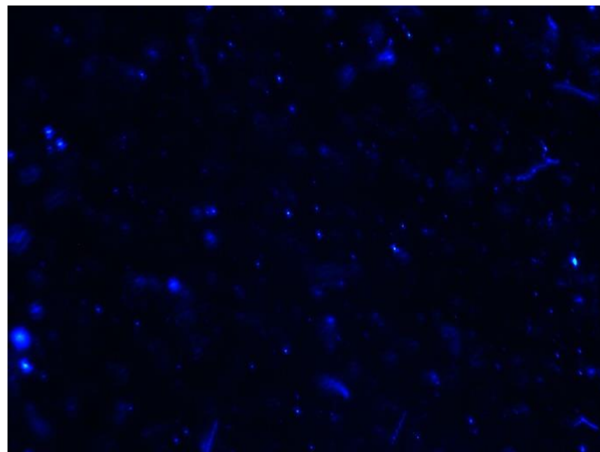


Figure 1B - Control under Fluorescence (no CD)

Figure 2A shows a sample of Borrelia under darkfield disinfected with 3 ppm of CD. There are no motile spirochetes or round bodies, only medium to large-size biofilms. This indicates 0% motility.

Figure 2B is the same sample but seen under Fluorescent-Darkfield microscopy using the Live-or-Dye™ viability stain. There is 0% motility with no spirochetes visible and no motile round bodies, but only highly phosphorescent masses of biofilms.

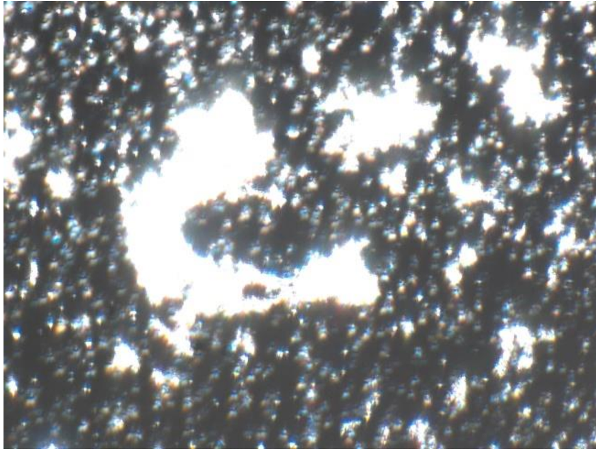


Figure 2A - 3 ppm CD under Darkfield

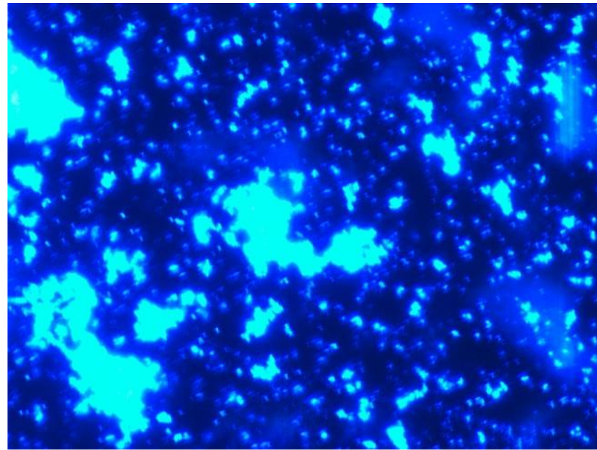


Figure 2B - 3 ppm CD under Fluorescence

Regrowth Kill Test (RKT) Results

In initial pilot experiments, we found that samples that had been disinfected with 2 ppm or higher and showed 0% motility, if they were placed back in the incubator in the Campypack for a few days, then the round bodies would come out of the biofilms and would be motile again. These initial pilot experiments were conducted using concentrations of CD from 1 – 5 ppm (Table 1, Graph 1).

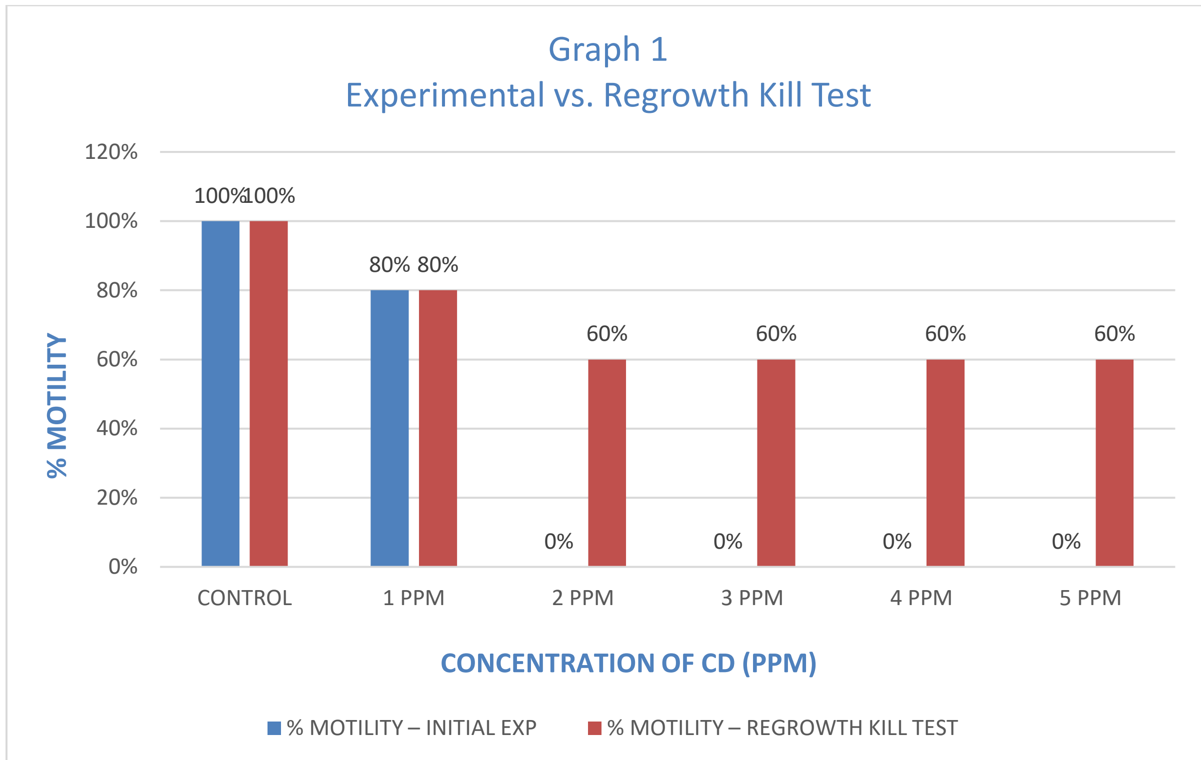
We, therefore, decided to conduct each experiment with what we termed the Regrowth Kill Test (RKT) by incubating the initial sample with the CD in an incubator at 37 degrees centigrade in the Campypack for 7-days. It appeared that the biofilms that are quickly formed as soon as the CD is added in the initial experiment, broke up and released small spirochetes and round bodies of different morphologies.

So, when 2 ppm of CD was initially added, biofilms were immediately formed and there was 0% motility detected. However, 7 days after incubating this sample, there was 60% motility observed under fluorescent-darkfield (Table 2, Graph 2). The same had occurred for concentrations of CD ranging from 2-5 ppm and above.

Table 1 – Percentage motility with CD in Initial Experiment vs Regrowth Assay 7-Days Later

CD (PPM)	**% MOTILITY – INITIAL EXP**	**% MOTILITY – REGROWTH KILL TEST**
CONTROL	100%	100%
1 PPM	80%	80%
2 PPM	0%	60%
3 PPM	0%	60%
4 PPM	0%	60%
5 PPM	0%	60%

N.B. All experiments were performed in triplicate, and these are the averages



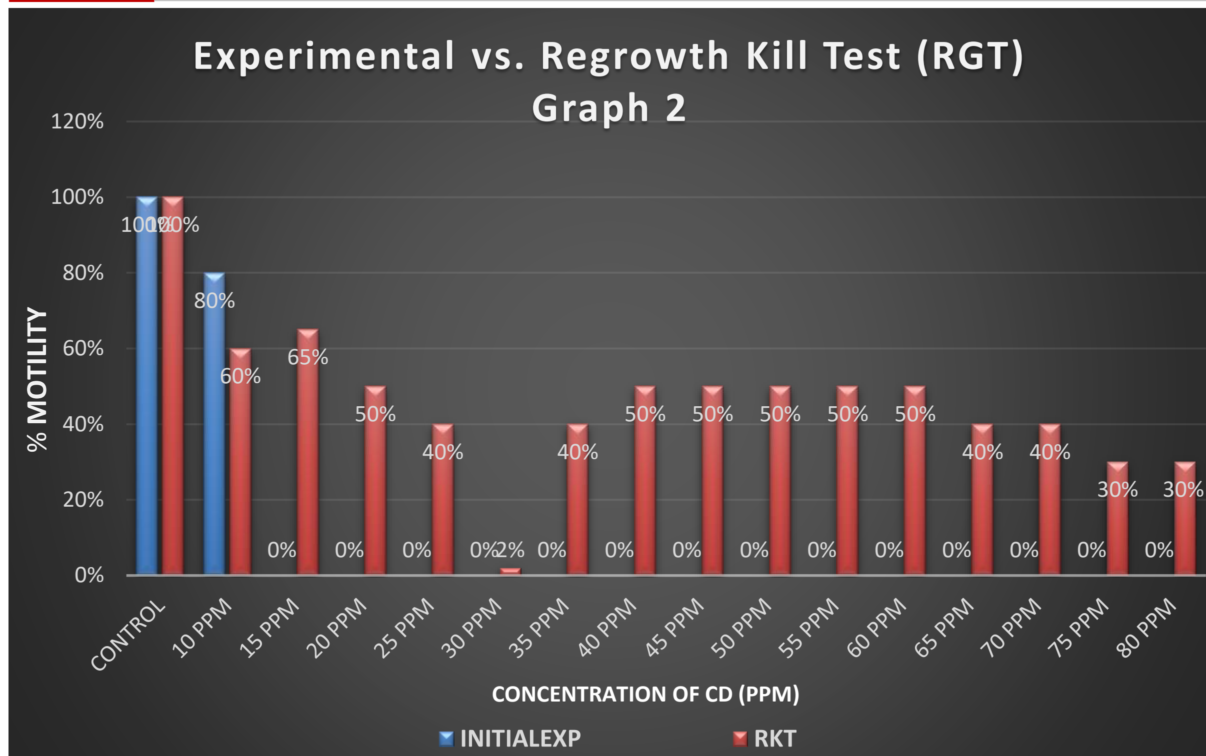
Further experiments increased the concentration of CD in 5 ppm increments, from 10 ppm up to 80 ppm. Table 2 shows that all concentrations used increased the motility of spirochetes and other round bodies in the RKT 7 days later (Table 2, Graph 2).

There is only one concentration of CD (30 ppm) that decreased the mobility to 2% during the RKT - these were only round bodies with no spirochetes of any size visible. This experiment was repeated in triplicate for all these concentrations and the results were the same as indicated in Table 2, Graph 2, and Figures 3A and 3B.

Table 2 – Percentage motility with CD in Initial Experiment vs Regrowth Assay 7-Days Later

CD (PPM)	% MOTILITY – INITIAL EXP	% MOTILITY – REGROWTH ASSAY
CONTROL	100%	100%
10 PPM	80%	60%
15 PPM	0%	65%
20 PPM	0%	50%
25 PPM	0%	20%
30 PPM	0%	2%
35 PPM	0%	40%
40 PPM	0%	50%
45 PPM	0%	50%
50 PPM	0%	50%
55 PPM	0%	50%
60 PPM	0%	50%
65 PPM	0%	40%
70 PPM	0%	40%
75 PPM	0%	30%
80 PPM	0%	30%

N.B. Averages of experiments performed in triplicate



Furthermore, all the above-mentioned experiments were repeated in triplicate using all three forms of chlorine dioxide (ClO_2) used, namely, CDSplus®,[38] CDSPure®, and CDS which is a pure chlorine dioxide gas bubbled into distilled water.

The results were not affected by the different types of chlorine dioxide, if the concentrations were correct.

Figure 3A shows a Borrelia sample disinfected with 30 ppm CD under darkfield. There are no spirochetes or round bodies visible, only large-sized biofilms.

Figure 3B is the same sample but seen under fluorescence-darkfield microscopy with stain. There is 2% motility of small round bodies, with no spirochetes visible, and high phosphorescent masses of biofilms

.

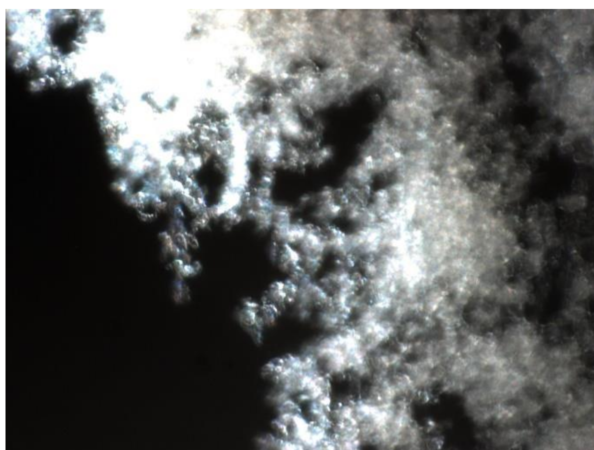


Figure 3A- 30 ppm CD under Darkfield (RKT)

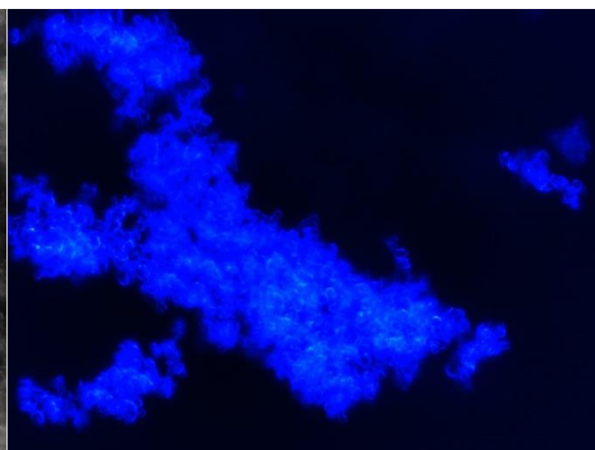


Figure 3B- 30 ppm CD under Fluorescence (RKT)

In the initial pilot experiments, small dosages of CD were tried to determine the effect on the motility of the various morphologies of Borrelia. It was found that 1 ppm CD still resulted in

over 80% motility. However, concentrations over 2 ppm or higher were found to result in 0% motility during the initial experiments (Table 1, Graph 1).

Discussion

These experiments have bought some interesting results to light that hopefully will enrichen the research literature and help clinicians treating Lyme Disease to make better clinical decisions.

It appears that chlorine dioxide is a powerful antimicrobial that can help to eradicate Borrelia morphologies in vitro. However, our experiments have shown that it appears to be dose-dependent and works best at concentrations of 30 ppm. The reason for this is not clear from this research.

In the initial experiments, even small dosages of 2 ppm or higher were seen to decrease the motility of the spirochetes and round bodies to 0%. However, if this sample was re-incubated for a further 7 days at 37 degrees centigrade (Regrowth Kill Test), then the biofilms would break up and release many motile round bodies with small spirochetes.

What we are observing in vitro is probably what is happening in the body, as health practitioners around the world are having difficulties eradicating Borrelia using either pharmaceutical or natural antimicrobials.

Borrelia is a pleomorphic[16,17,18] microorganism and this is probably the reason why 36-63% of patients treated with these antibiotics still suffer from persistent symptoms,[9] a condition that has been termed Post Treatment Lyme Disease (PTLD).

Internal changes such as pH, temperature, oxidative stress, change of media composition, acidity/alkalinity, concentrations of salts, sugars, or other organic compounds or antibiotic treatments can all change the morphology from spirochetes to RB such as granules, cysts, spheroplasts or vesicles.[73-81] Additionally, various bacteria can also aggregate into biofilms.[22,23,24,25]

This research confirms what many other scientists have mistaken as a decrease in pathogen viability when there is a transformation of the motile spirochaetes into RBs. We have shown here in vitro that motile RBs can transform into active growing reproductive spirochetes when the conditions are favourable.[64, 70-73,74-76,80,82]

The dose-dependent results that we have obtained using CD with Borrelia may be a result of persisters.[85-86] When the concentration of antimicrobials exceeds a certain threshold, only persister cells survive.[87] It is possible for persisters to undergo a change to "reverters" (replicating forms) under the right conditions, therefore causing relapse and chronic infection.[84-87]

Immersing the Borrelia morphologies in a 30 ppm concentration of CD seems in vitro to prevent the persisters from transforming to reverters. This was the only concentration that resulted in a near complete disinfection of the Borrelia morphologies (only 2% motile after the 7-days of the RKT).

There were many experiments performed using concentrations of CD from 1 – 80 ppm. Even though concentrations of 2 ppm or greater would apparently result in complete eradication as soon as the CD was added, when the sample was placed back in the incubator for 7 days, the Borrelia morphologies became motile again.

The near complete disinfection was obtained at 30 ppm of CD after the 7-day RKT. This resulted in about 98% eradication of all morphologies including the spirochetes and the round bodies.

Conclusions

It is interesting to note that this research has found that 30 ppm Chlorine dioxide is the optimal level required in vitro to eliminate motility in the initial experiments as well as the Regrowth Kill Test seven days later.

It would certainly be interesting for other researchers and clinicians to try implementing a clinical trial using this dosage of CD with patients diagnosed with Lyme's Disease and Borrelia burgdoferi bacteria.

There are sealed, sterile ampoules of CD (CDSPure®) – 5ml ampoules containing 2,990 ppm CD that can be used directly IV, or alternatively by mouth using CDSplus® - 100 ml bottle containing 2,990 ppm.

Clinical trials would need to be completed to gain clinical experience in what works best, however, extrapolating from the in vitro research it would be reasonable to use the following dosages.

For the average 70 kg adult, it would require one ampoule (5 ml = 2,990 ppm) of the CDSPure® in a 500ml saline IV infusion (1:100 dilution). Alternatively, 20 ml of the CDSplus® in a 1 litre bottle of water drunk over a few hours. It is likely that this would need to be repeated a couple of times per week until the level of Borrelia is eradicated, without giving the opportunity to regrow.

Various clinicians treating Lyme's disease have postulated that the round bodies as well as the spirochaetes can penetrate muscles and organs and "hide" there, protected from any type of antimicrobial. The chlorine dioxide molecule, however, is so small at 0.124 nm, that it is smaller

than the smallest virus and can therefore penetrate through the blood-brain barrier and other tissues of the body.

It is the hope of this author that other clinicians and researchers will take these results and build on them. Given that there are thousands of people suffering from Lyme disease and related symptoms[111], it would be wise to set up a clinical trial using the results of this research to determine the efficacy of chlorine dioxide at a specific concentration on Borrelia-related symptoms. The author would welcome such interest from other clinicians.

Conflict of Interest: The author has no conflict of interest to declare.

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