Comparing the Anti-Microbial Effect of Penicillin to that of Chlorine Dioxide on Three Opportunistic Pathogens

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Independent Research Project for Bio489

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Abstract

Introduction: The discovery of penicillin is a watershed in the history of medicine. Today, antibiotics are a part of everyday life. However, some speculate that society may return to the pre-antibiotic era if the increasing emergence and spread of antibiotic resistant bacteria cannot be slowed. Resistant genes are often carried on plasmids and can spread quickly via horizontal gene transfer. Opportunistic pathogens are key species for developing antibiotic resistance. These pathogens are ubiquitous in nature and live symbiotically on or in humans. After a major infection, transient colonies often remain to spread antibiotic resistance. Because of the growing concern over antibiotic resistance, many people have started looking for natural alternatives to antibiotics. This paper proposes that chlorine dioxide (ClO2) should be considered as an alternative to common antibiotics. ClO2 disinfects via oxidation and is highly selective due to its one-electron transfer mechanisms. Chlorine dioxide is currently used as a common disinfectant in: drinking water, dental practices, and as an aerosol. This paper will compare the anti-microbial effect of penicillin to that of chlorine dioxide on *Enterococcus faecalis, Staphylococcus epidermidis* and *Bacillus subtilis,* three opportunistic pathogens. *Methods:* Part one of the experiment was a phenol coefficient test which was completed to determine the minimum inhibitory concentration (MIC) of chlorine dioxide. In part two, a standard disk diffusion method was completed using the minimum inhibitory concentrations of penicillin and ClO2 on each disk. *Results and Conclusion*: In part one the MIC of chlorine dioxide was found to be a 1:100 dilution. This concentration is on par with the MIC found for phenol and indicates that ClO2 is as effective as phenol in inhibiting bacterial growth. In part two ClO2 was found to be less effective than penicillin against the three opportunistic pathogens. These results may be erroneous however as, part two, trial two of the experiment, shows evidence for a flawed experimental design. The standard disk diffusion method may not be an accurate way of measuring the antibiotic effectiveness of ClO2 as liquid chlorine dioxide likely dries in the incubator and loses its inhibitory effect. Further avenues of research should be explored to more accurately test the effectiveness of ClO2 on pathogens.

Introduction

Penicillin

Prior to the development of antibiotics, many infections were fatal. The discovery of penicillin is considered a watershed in the history of medicine and has changed this field drastically (Ligon 2004; Hamdy 2006).

Penicillin, the first antibiotic used on a large scale, was discovered by accident (Kingston 2000). Alexander Fleming is credited with penicillin's discovery. In 1928, Fleming found a blue-green mold on an old petri dish. What he found interesting was the clear zone around the mold where the *Staphylococcus* did not grow. This clear zone led Fleming to recognize the mold as a potential antibacterial agent. (Bennett and Chung 2001)Fleming called the "mold broth filtrate" penicillin but soon lost interest in it. Luckily, when culturing swabs from patients' throats, Fleming found that this penicillin producing mold (*Penicillium notatum*) would inhibit the growth of other microbes in the culture and allow *Haemophilus influenza* to flourish; this allowed *H. influenza* to be identified as the cause of the disease. Therefore, Fleming kept this rare strain of mold in culture to be used for identifying influenza (Bentley 2009; Shama 2009; Kingston 2000).

In 1937, the scientists Howard Florey and Ernst Chain became interested in penicillin. It is through the meticulous work of these two scientists that penicillin was finally extracted and purified. In 1940, Florey and Chain published a paper regarding the production and purification of penicillin (Ligon 2004; Bentley 2009; Bennett and Chung 2001; Chain and others 1940).

The needs of World War II pushed the Allied forces to optimize penicillin production. It was observed that many soldiers died from infection rather than battle. Penicillin was kept as a secret from German forces, because it was seen as a major advantage (Shama 2009). Eventually, a system for mass production of penicillin was established. The fungus was grown through deep tank fermentation, using lactose and corn

steep liquor (Bennett and Chung 2001). The increased efficiency in the production of penicillin is seen when comparing the American production of 1941 to 1943. In 1941, the United States did not have enough penicillin to treat a single patient. In 1943, the supply of penicillin was so great it satisfied the demands of the entire Allied Armed Forces (Ligon 2004).

In 1945, Florey, Chain and Fleming shared the Nobel Prize for their work on penicillin. Unfortunately, the media at the time focused mostly on Fleming, while Florey and Chain were ignored. This error led to resentment on the part of Florey and Chain. Even today, when people are asked about the development of penicillin, most people only mention Fleming (Bennett and Chung 2001).

Penicillin is in a class of antibiotics known as Beta-lactam antibiotics (β-lactams). Today, this is a large class of antibiotics that are named after the structure they all share, a β-lactam ring. These antibiotics work by inhibiting bacterial cell wall synthesis. Some common β-lactams are as follows: penicillin, amoxicillin, methicillin, and oxacillin (Banoee and others 2010; Smith and Jarvis 1999).

The β-lactam structure mimics the structure of the terminal portion of peptidoglycan; this portion of peptidoglycan is cross linked into the polymeric structure. The overall structure imparts rigidity to the cell wall. The enzymes that catalyze crosslinking are known as penicillin binding proteins (PBP). β-lactam antibiotics bind to the active site of the PBP's and irreversibly inhibit these enzymes from completing the cell wall structure (Kotra and Mobashery 1999; Kotra and Mobashery 1998; Smith and Jarvis 1999).

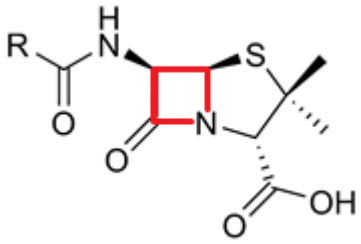

Fig 1. Core structure of B-lactam antibiotics, red box indicates β-lactam ring (Kotra and Mobashery 1999).

Antibiotic Resistance

Over sixty years have passed since humans began using antibiotics for widespread use. Unfortunately, society may return to the pre-antibiotic era if the increasing emergence and spread of antibiotic resistant bacteria cannot be slowed (Bennett and Chung 2001; Charles and Grayson 2004; Davies and Davies 2010). Bacterial resistance to penicillin was discovered as early as 1940; the first clinical case of penicillin resistant was recorded in 1941. The semi-synthetic antibiotic methicillin was developed to address penicillin resistance in 1959. Unfortunately, the first case of methicillin resistant bacteria was documented two years later (Smith and Jarvis 1999).

A wide range of biochemical and physiological mechanisms are responsible for resistance (Davies and Davies 2010). At the most basic level, gram negative bacteria are naturally resistance to penicillin because the outer membrane protects peptidoglycan from penicillin's effects. Penicillin (and other β-lactam antibiotic) resistance in gram positive bacteria is caused by the action of a group of enzymes known collectively as β-lactamase's. β-lactamase is an enzyme that was derived from penicillin binding proteins and acts to effectively hydrolyze the β-lactam ring (Jayaraman 2009; Stein 2011). There are several hundred types of β-lactamase enzymes and many classification systems have been proposed (Korta and Mobashery 1999; Ambler 1980; Bush and others 1995).

β-lactamases can sometimes be inhibited by a combination of β-lactam antibiotics and inhibitory compounds such as clavulanic acid. These compounds act as suicide inhibitors of β- lactamase and allow the antibiotic to retain some degree of effectiveness. Unfortunately, resistance to these antibiotic/inhibitor compounds developed soon after their first use (Wong and others 2003; Kotra and Mobashery 1998).

Resistant genes (such as β-lactamase) are often carried on plasmids. Horizontal gene transfer facilitates easy movement of plasmids between bacteria of the same species and between other species, allowing resistant genes to spread quickly (Stein 2011). In 1946, it was estimated that 6% of *Staphylococcus aureus* strains carried β-lactamase; by 1950 that number had jumped to 50%. Currently 90-95% of *S. aureus* strains are resistant to β-lactam antibiotics due to the possession of some form of β-lactamase (JiYeon 2009; Stein 2011).

Despite rampant resistance, β-lactam antibiotics are still the most commonly used antibiotics world-wide, mainly due to their broad spectrum activity and low toxicity (Wong and others 2003). However, there are many other types of antibiotics such as tetracyclines, macrolides and quinolones. Unfortunately, all antibiotics have encountered resistance of some kind (Stein 2011; Davies and Davies 2010).

There are four major types of antibiotic resistance common to bacteria and all of them can be found associated with β-lactams. The first type of resistance occurs when the bacteria modifies or inactivates the antibiotic, as seen with β-lactamase. The second type, the most common, occurs when the bacteria pumps the antibiotic back out of the cell via efflux pumps. The third and fourth types occur when the bacteria structurally changes the antibiotic target or acquires a second target that is unaffected by the antibiotic, causing the antibiotic to lose effectiveness (Stein 2011).

Antibiotic resistance is millions of years old (Jayaraman 2009; Davies and Davies 2010). Many microbes produce antibiotics to prevent the growth of competitors, their competitors in turn, develop resistance to these antibiotics. This produces a cycle of co-evolution that typically develops slowly over many

years; unfortunately, humans have radically sped up this process (Jayaraman 2009; Davies and Davies 2010). The irresponsible use of antibiotics is at the core of the problem with antibiotic resistance. Problems began as soon as penicillin was released to the public. For nearly 10 years penicillin was available over the counter without a prescription. Now that antibiotics require a prescription, doctors prescribe them for infections that do not require antibiotics. A survey done in 1992 showed that 21% of antibiotic prescriptions are given for conditions not requiring them (Davies and Davies 2010; Gonzales and others 1997).

Not only do humans use antibiotics inappropriately, but they are also incorporated into livestock feed unnecessarily. It is estimated that over half of the antibiotics produced are fed to livestock (Davies and Davies 2010; Stein 2011). In the 1950's, it was discovered that giving antibiotics to healthy animals increased their growth rate. Unfortunately, this practice has contributed to the world's problem of antibiotic resistance since resistant pathogens can be transmitted from animals to humans (Stein 2011; Martinez 2009). Overuse and misuse of antibiotics in both humans and animals needs to be stopped on a global scale to prevent the return of pre-antibiotic medicine (Smith and Jarvis 1999).

Humans dump tons of toxic chemicals into the environment each year. Many people do not include antibiotics in the category of toxic chemicals, but they actually cause considerable harm to the ecosystem. With the introduction of resistant genes with expanded potential, we could be drastically changing microbial populations and hence entire ecosystems (Martinez 2009; Aust and others 2008; Cordova-Kreylos and Scow 2007; Dantas and others 2008). Resistant genes have even been found in very remote areas of the planet. It has been hypothesised that this phenomenon occurs due to occasional contact of organisms from antibiotic-exposed areas with these remote regions; these organisms could be migratory birds, animals or humans (Pallecchi and others 2008; Stein 2011). The dumping of antibiotics into the ecosystem is a major concern for scientists; a particularly extreme example is the daily dumping of over 50kg of ciprofloxacin into a river near

Hyderabad, India by the pharmaceutical manufacturer Patancheru Enviro Tech Ltd. (Davies and Davies 2010; Pallecchi and others 2008; Fick and others 2009).

All of the misuse, overuse, and dumping of antibiotics provides a powerful force behind the natural selection of resistant genes (Davies and Davies 2010; Smith and Jarvis 1999). It has been shown that resistance rates are directly proportionate to the extent of antibiotic use in the area (Stein 2011; Goossens and others 2005).

Many viable solutions to the global problem of resistance have been proposed. First and foremost, there needs to be strict limitations upon the amount of antibiotics used, and narrow spectrum antibiotics should be used whenever possible. Further, antibiotics should not be provided for prophylactic reasons. Finally, methods of breaking down these antibiotic compounds should be put into practice in waste treatment centres to prevent dumping (Martinez 2009; Smith and Jarvis 1999).

Antibiotic resistance will occur no matter what is developed to combat it. Antibiotic resistance is a natural process that needs to be mitigated (Jayaraman 2009). Many new antibiotics have been developed with naive hope. When synthetic antibiotics were developed, (such as fluoroquinolones) it was assumed that resistance would not develop. Similarly, when vancomycin was developed, it was assumed that resistance would not develop because the drug binds non-covalently to the cell wall. Resistant bacteria developed for both fluoroquinolones and vancomycin. These two cases provide evidence that bacterial resistance can develop to whatever antibiotics are developed to counter them (Jayaraman 2009; Davies and Davies 2010).

There is a decline in the development of new antibiotics. Pharmaceutical companies do not find it beneficial to develop new antibiotics. The development of new antibiotics is hardly worth the time and money required when bacteria typically develop resistance within two years of reaching the market (Charles and Grayson 2004). Antibiotic development has declined to about 1.6 new drugs annually as of the year 2000; this is not enough to stay ahead of the growing number of antibiotic resistant bacteria. It is estimated

that there are currently 20,000 antibiotic resistant genes available to bacteria. New antibiotics need to continually be developed at an appropriate rate to maintain current standards of medicine (Davies and Davies 2010; Stein 2011). One solution proposed by Charles and Grayson (2004) is to create greater cooperation between governments, academic institutions and pharmaceutical companies in sharing the costs required to develop new drugs. Recently, it has become difficult to produce new, safe antibiotics because the therapeutic window for many bacteria is becoming quite small. A small therapeutic window means that the amount/type of antibiotic required to kill the pathogen will potentially harm or kill the human as well (Merriam Webster Online Dictionary 2012; Charles and Grayson 2004). In the past, many antibiotics have been discarded early in their development because of highly toxic effects such as blindness and organ damage. Unfortunately, due to closing therapeutic windows, many highly toxic antibiotics are now being reconsidered for human use as their toxic effects are considered better than death (Charles and Grayson 2004).

Opportunistic Pathogens

Opportunistic pathogens are key species for the development of antibiotic resistance. These pathogens are ubiquitous in nature and live symbiotically on and in human beings, but can cause disease if given the chance. Because of their close relationship with humans, these pathogens acquire resistant genes with greater ease than other bacteria. After a major infection, transient colonies of opportunistic pathogens often remain to spread antibiotic resistance to other bacteria (Devirgiliis and others 2011).

Enterococcus faecalis

Enterococcus faecalis is a gram positive cocci that is part of the normal gut flora in humans. *Enterococcus spp.* have natural tolerance for adverse environmental conditions such as the ability to grow in 6.5% sodium chloride solutions, 40% bile salt solutions, at a pH as high as 9.6, and temperatures ranging from

10˚C to 45˚C (Sood and others 2008; Ogier and Serror 2008). Because of these unique abilities, *E. faecalis* have been used in the fermentation process of many foods, primarily cheese and sausage (Lenz and others 2010). Despite *E. faecalis* widespread use as a fermentation agent, and even as a probiotic, it is questionable whether *E. faecalis* is safe for use and consumption (Ogier and Serror 2008). Since the 1990's *E. faecalis* has been labeled as an opportunistic pathogen. *E. faecalis* is harmless to healthy individuals but causes serious infections in the immuno-compromised population. *E. faecalis* is rampant in hospitals and causes many nosocomial infections. In fact, 80% of all *Enterococcus spp.* infections are caused by *E. faecialis* (Ogier and Serror 2008). In the United States, *E. faecalis* is the third leading cause of septicemia, the second leading cause of urinary tract infections, and the third leading cause of endocarditis. *E. faecalis* endocarditis is thought to arise from endogenous bacteremia originating from the individuals normal gastrointestinal *E. faecalis* colony (Lenz and others 2010; Ogier and Serror 2008).

The average mortality rate for an enterococcal infection is between 20 to 30% but has been seen to be as high as 68% (Ogier and Serror 2008; Sood and others 2008). These high mortality rates are because of *enterococci's* intrinsic tolerance to many classes of antibiotics, including beta-lactams. Further*, E. faecalis* has acquired many antibiotic resistance genes that have spread throughout its populations (Ogier and Serror 2008). Today, *E. faecalis* has been found to carry resistant genes to just about every class of antibiotic known (Ogier and Serror 2008; Aslam and others 2012; Sood and others 2008).

Enterococcus spp. biggest threat is not the infections it causes but the fact that it is a major reservoir for antibiotic resistance genes. These genes can be carried by a normal gut colony in healthy individuals and transferred to more serious pathogens upon infection or via fecal contamination. Due to enterococcus high rate of conjugation, transfer of genetic material is very likely to occur (Aslam and others 2012; Lenz and others 2010). The most classic case of gene transfer between *Enterococcus spp.* and another pathogen is between *Enterococcus spp.* and *Staphylococcus aureus*. Vancomycin was developed in 1958, by 1988 the

occurrence of vancomycin resistant *Enterococcus* (VRE) was common. Scientific evidence supports the notion that *Enterococci* transferred its vancomycin resistance gene (vanA gene) to *Staphylococcus aureus* strains in 2002 and gave rise to vancomycin resistant *S. aureus* (VRSA) (Jayaraman 2009; JiYeon 2009; Shinefield and Ruff 2009). *S. aureus* is the most common and virulent pathogen today and VRSA is of great concern to the medical community (Jayaraman 2009).

Transfer of resistance genes between *E. faecalis* and other pathogens is feared to happen again with the gene for resistance to the streptogramin antibiotic quinupristin/dalfopristin (QD). Since the rise of VRE, QD has been used to treat VRE infections. Unfortunately, streptogramins (and hence QD) are very similar in structure and function to virginiamycin, a common antibiotic used in agriculture to promote animal growth. If resistance to virginiamycin develops in enterococci species, cross resistance is likely to occur to streptogramins as well. Streptogramin resistant *Enterococcus* (SRE) would be a great threat to public health as there is no known treatment (Smith and others 2003).

Much study has been conducted concerning what causes *Enterococcus faecalis* to change from a commensal bacteria to a pathogen. Many virulence associated genes have been found in *E. faecialis* that encode for: extracellular matrix proteins, resistance to macrophages, immune system evasion, and cell and tissue damage (Aslam and others 2012; Ogier and Serror 2008). Unfortunately, to date, no information is available about the distribution and diversity of these virulence associated genes. Further, the factors that are involved in the switch from commensalism to pathogenicity in *E. faecalis* still remain unclear (Lenz and others 2010).

Staphylococcus epidermidis

Staphylococcus aureus is the most common and virulent pathogen in hospitals worldwide today. A close cousin *to S. aureus* is *Staphylococcus epidermidis*. *S. epidermidis* is a gram positive non-motile bacteria

that is a permanent and ubiquitous colonizer of human skin (Kiedrowski and Horswill 2011; Otto 2009). *S. epidermidis* rarely causes infections in healthy individuals, and has the probiotic function of preventing serious pathogens from colonizing the skin (Otto 2009). *S. epidermidis* is described as an opportunistic pathogen because it will cause infections if the skin is broken; these infections are usually seen in immune-compromised people. However, *S. epidermidis* is best known for causing infections disseminated by indwelling medical devices such as catheters, prosthetic joints, cardiac devices, CNS shunts, and vascular grafts (Rolo and others 2012; Otto 2009).

Unlike its cousin *S. aureus, S. epidermidis* does not aggressively attack its host with toxins. *S. epidermidis* infections are normally mild but chronic (Otto 2009). Treating *S. epidermidis* is very difficult due to its acquisition of many immune system evasion mechanisms. *S. epidermidis* has exopolymers called poly-y-glutamic acid (PNG) and Poly-N-acetylglucosamine (PNAG); these exoploymers protect the cell from antibody detection and provide resistance to neutrophils, and antimicrobial peptides (AMP). *S. epidermidis* also has the ability to form a biofilm. The biofilm protects the bacteria from the immune system and from antibiotics by stopping these agents from reaching the bacteria cells (Otto 2009; Kiedrowski and Horswill 2011).

S. epidermidis, like other pathogens, has acquired many antibiotic resistant genes. However, unlike *S. aureus*, very little attention is paid to *S. epidermidis* infections due to their mild nature; therefore, intense hygienic measures are not taken with *S. epidermidis* infections. This lack of attention has caused the high rates of antibiotic genes found in *S. epidermidis* strains. It has been estimated that 75-90% of *S. epidermidis* strains carry methicillin resistance and that 90-100% of strains carry β-lactam resistance. No vancomyin resistant *S. epidermidis* strains have been found to date; however, a few cases of vancomyin tolerance have been reported (Habes and others 2012; Ziebuhr and others 2006; Begun and others 2011).

Because intense hygienic measures are not followed for *S. epidermidis,* it has become a reservoir for novel resistance genes that can be easily transmitted to *S. aureus* or other serious pathogens (Ziebuhr and others 2006; Habes and others 2012). Ziebuhr and others (2006) suggests that we treat *S. epidermidis* infections more seriously and implement more intense hygienic measures to keep *S. epidermidis* under control and from developing vancomycin resistance as well.

Bacillus subtilis

Bacillus subtilis is a gram positive, aerobic, endospore former. It is not only a common soil bacteria, but has been found in many places including: the rhizophere of plants, fresh and salty water, and as part of the intestinal flora of many animals such as sow-bugs, cattle and humans (Maughan and Auwera 2011; Logan 2011). *B. subtilis* is one of the best studied soil microorganisms and hence is the type species of the Bacillus genus (Lopez and others 2009; Logan 2011). *B. subtilis* is unique in that it is capable of exhibiting many different growth characteristics. Not only can *B. subtilis* form endospores, it can also alternate between a motile and sessile states. Because of *B. subtilis* natural competent abilities it has been used for enzyme production, especially production of protease and lipases with have a high market value (Dubnau 2010; Vojcic and others 2012).

Of the Bacillus genus*, B. cereus* attracts the most attention with its ability to cause severe local infections or systematic infections such as endophthalmitis and septicaemia. *B. cereus* is also a common culprit of food poisoning, causing two kinds of gastrointestinal disease, (Diarrhoeal and/or emetic) determined by the strain present (Logan 2011). *Bacillus subtilis* is generally recognized as safe by the FDA, however, it has recently come to light that *B. subtilis* is also capable of producing food borne illnesses. This fact has called in to question the safety of using *B. subtilis* in food fermentation such as the oriental fermentation of natto in Japan (Arnesen and others 2008).

Very little is known about the virulence factors of *B. subtilis* or the percentage of food borne illnesses it causes. It has been found that several *B. subtilis* strains encode for dangerous heat stable toxins such as lichenysin A, surfactin, and amylosin (Apetroaie-Constantin and others 2009; From and others 2005). Like *B. cereus*, *B subtilis's* ability to form endospores makes it resistant to many disinfecting processes such as heat, radiation, desiccation, and physical stress, and hence very difficult to get rid of in hospitals and food production facilities (Arnesen and others 2008).

Though *B. subtilis* is not a major threat to humans, it still plays a crucial role in our problems with antibiotic resistance. Because of its ubiquity and presence in the intestines of animals and humans, *B. subtilis* is a reservoir for antibiotic resistant gene. With its natural complement abilities, *B. subtilis* can easily pick up antibiotic resistance genes from the environment and transfer them to more virulent pathogens (Schlesinger and others 2011; Lopez and others 2009).

Chlorine Dioxide

Because of the growing concern over antibiotic resistance, many people have started looking for natural alternatives to traditional antibiotics such as essential oils, honey and zinc oxide (Bulut and others 2009; Tajik and others 2008; Banoee and others 2009). I propose that chlorine dioxide be considered as an effective alternative to common antibiotics.

Chlorine dioxide (ClO_2) has been shown to be: bactericidal, viricidial, sporocidal, cysticidal, algicidal, and fungicidal (Grootveld and others 2001; Callahan and others 2010; Pavoni and others 2004; Meneghin and others 2008; EPA 1999; Rajkovic and others 2009). ClO_2 is highly explosive when made in concentrations above 10% (100g/L). Luckily, many studies have shown that a solution of ClO_2 well below 10% is effective for disinfection (EPA 1999). Callahan and others (2010) found that ClO_2 can create a $\log_{10}$ reduction of bacterial cells with just a 0.75mg/L solution. Pavoni and others (2004) found that ClO_2 created a 4 log reduction of

viable viruses with a 1mg/L solution. Further, Stampi and others (2002) found that *E. coli* cell count could be reduced 99.97% with the usage of 2.2mg/L of ClO_2. Hofmann and others (1997) showed that ClO_2 is even effective against parasites; a dose of 1.5mg/L was shown to produce a 3 log reduction of *Giardia* protozoan.

ClO_2 disinfects via oxidation and is highly selective due to its one-electron transfer mechanisms. It can be maintained in aqueous solutions for long periods of time when stored in a dark, cold (4˚C) room (Wang and others 2011). In solution, ClO_2 is yellow in color and smells like chlorine. EPA registered chlorine dioxide in its liquid form in 1967 for use as a disinfectant and sanitizer (US. Department of Health and Human Services 2004).

The creation of ClO_2 can be accomplished in many ways, most commonly by mixing sodium chlorite ($NaClO_2$) with either gaseous chlorine or an acid compound. Table 2 (below) shows the various ways of producing ClO_2. The Acid-Chlorite method is the easiest method for production; however it only creates an 80% yield.

GENERATOR TYPE	MAIN REACTIONS Reactants, byproducts, key reactions, and chemistry notes	SPECIAL ATTRIBUTES
ACID-CHLORITE: (Direct Acid System)	$4HCl + 5NaClO_2 \rightarrow 4ClO_{2(aq)} + Cl^-$ • Low pH • ClO_3^- possible • Slow reaction rates	Chemical feed pump interlocks required. Production limit ~ 25-30 lb/day. Maximum yield at ~80% efficiency.
AQUEOUS CHLORINE-CHLORITE: (Cl_2 gas ejectors with chemical pumps for liquids or booster pump for ejector water).	$Cl_2 + H_2O \rightarrow [HOCl / HCl]$ $[HOCl/HCl] + NaClO_2 \rightarrow$ $ClO_{2(g)} + H_2OCl^- + NaOH + ClO_3^-$ • Low pH • ClO_3^- possible • Relatively slow reaction rates	Excess Cl_2 or acid to neutralize NaOH. Production rates limited to ~ 1000 lb/day. High conversion but yield only 80-92% More corrosive effluent due to low pH (~2.8-3.5). Three chemical systems pump HCl, hypochlorite, chlorite, and dilution water to reaction chamber.
RECYCLED AQUEOUS CHLORINE OR "FRENCH LOOP"™ (Saturated Cl_2 solution via a recycling loop prior to mixing with chlorite solution.)	$2HOCl + 2NaClO_2 \rightarrow 2ClO_2 + Cl_2 + 2NaOH$ • Excess Cl_2 or HCl needed due to NaOH formed.	Concentration of ~3 g/L required for maximum efficiency. Production rate limited to ~ 1000 lb/day. Yield of 92-98% with ~10% excess Cl_2 reported. Highly corrosive to pumps; draw-down calibration needed. Maturation tank required after mixing.
GASEOUS CHLORINE-CHLORITE (Gaseous Cl_2 and 25% solution of sodium chlorite; pulled by ejector into the reaction column.)	$Cl_{2(g)} + NaClO_{2(aq)} \rightarrow ClO_{2(aq)}$ • Neutral pH • Rapid reaction • Potential scaling in reactor under vacuum due to hardness of feedstock.	Production rates 5-120,000 lb/day. Ejector-based, with no pumps. Motive water is dilution water. Near neutral pH effluent. No excess Cl_2. Turndown rated at 5-10X with yield of 95-99%. Less than 2% excess Cl_2. Highly calibrated flowmeters with min. line pressure ~ 40 psig needed.
GASEOUS CHLORINE-SOLIDS CHLORITE MATRIX (Humidified Cl_2 gas is pulled or pumped through a stable matrix containing solid sodium chlorite.)	$Cl_{2(g)} + NaClO_{2(s)} \rightarrow ClO_{2(g)} + NaCl$ • Rapid reaction rate • New technology	Cl_2 gas diluted with N_2 or filtered air to produce ~8% gaseous ClO_2 stream. Infinite turndown is possible with >99% yield. Maximum rate to ~1200 lb/day per column; ganged to >10,000 lb/day.
ELECTROCHEMICAL (Continuous generation of ClO_2 from 25% chlorite solution recycled through electrolyte cell)	$NaClO_{2(aq)} \rightarrow ClO_{2(aq)} + e^-$ • New technology	Counter-current chilled water stream accepts gaseous ClO_2 from production cell after it diffuses across the gas permeable membrane. Small one-pass system requires precise flow for power requirements (Coulombs law).
ACID/PEROXIDE/CHLORIDE	$2NaClO_3 + H_2O_2 + H_2SO_4 \rightarrow 2ClO_2 + O_2 + NaSO_4 + H_20$	Uses concentrated H_2O_2 and H_2SO_4. Downscaled version; Foam binding; Low pH.

Table 2. Commercial Chlorine Dioxide Generation. *Adapted from Gates 1998* (EPA 1999).

Drinking water is often disinfected with ClO_2. Water treatment plants across the world have implemented ClO_2 as a replacement for chlorine because it does not produce harmful by-products such as THM's (trihalomethances) and HAA's (haloacetic acids) (Navalon and others 2008; Gordon 2001; Harris 2001). Furthermore, ClO_2 is less pH dependent than chlorine and can be effective at pH ranges of 4 to 10 (Meneghin

and others 2008). Today, about 700 to 900 public water systems in the United States use ClO_2 (Wang and others 2010).

Dentists use ClO_2 as an oral rinse and as a means of getting rid of biofilms on dentist tools (Grootveld and others 2001). ClO_2 can even be used as an effective surface disinfectant when applied as a mist (Callahan and others 2010).

Antibiotics such as Tetracyclines, Fluoroquinolones and Beta- lactams that have built up in the environment can be broken down by ClO_2. This means that ClO_2 can not only be used as a replacement antibiotic, but can also break up excess traditional antibiotics in the environment that contribute to widespread antibiotic resistance (Wang and others 2011; Wang and others 2010). ClO_2 leaves the body via urine, and since chlorine dioxide breaks down in sunlight, it will not build up in the environment (US. Department of Health and Human Services 2004).

Unfortunately, very little work has been conducted on the effects of ClO_2 on humans when ingested. Lubbers and others (1982) did a 3 phase experiment on adult males where upwards of 24mg/L of ClO_2 were ingested. The experimenters found no clinically important impact upon the health of the subjects. Lubber and others did note that long term negative impacts were not assessed and further research should be conducted. Michael and others (1981) found no adverse health effects in people living in a rural village exposed to ClO_2 concentrations ranging from 0.25 to 1.11mg/L over a 12 week period. The authors again concluded that long term negative impacts were not assessed and further research should be conducted.

Inhalation of ClO_2 is not recommended as ClO_2 can act as a respiratory and ocular irritant when in its gaseous form. Limited human studies have been conducted; however, one animal study showed that exposure to 2.5 ppm of ClO_2 for 7 hours a day for 30 days resulted in: lymphocytic infiltration of the alveolar spaces, alveolar vascular congestion, hemorrhagic alveoli, epithelial erosions, and inflammatory infiltrations

of the bronchi. The above study gives reason to explore ClO_2 only as an internal antibiotic that is to be ingested (Paulet and Desbrousses 1970; US. Department of Health and Human Services 2004).

By-products of ClO_2 have been linked to toxic effects. Fifty to seventy percent of ClO_2 breaks down into chlorite, and thirty percent into chlorate (EPA 1999). Both of these chemicals have been linked to anemia and the inhibition of normal thyroid function (Harrington and others 1985; Stampi and others 2002). However, Meneghin and others (2008) showed that chlorite and chlorate pose no risk to human health at low concentrations. EPA (1999) suggests no more than 1.4mg/L of ClO_2 be used for treating drinking water as levels above 1.4mg/L allow the chlorite and chlorate concentrations to exceed maximum safe doses of 1mg/L. Lubber and others (1982) suggest short term doses of upwards of 24mg/L do not have a negative effect on human health. However, further research needs to be conducted concerning the use of chlorine dioxide as an internal antibiotic.

It should be noted that not all individuals respond to ClO_2 in the same way. Michael and others (1981) suggested that people with glucose-6-phosphate dehydrogenase deficiency may be more sensitive to chlorine dioxide and develop anemia more rapidly with small doses. Glucose-6-phosphate dehydrogenase deficiency occurs in 10% of the African American population. Further, pregnant or nursing women should not be exposed to ClO_2 as animal studies have shown that ClO_2 causes delays in brain development in rats and rabbits (US. Department of Health and Human Services 2004).

Conclusion

The discovery of penicillin is a watershed in the history of medicine. Today, antibiotics are a part of everyday life. However, it is speculated that our society may return to the pre-antibiotic medicine if something is not done to curb the increasing emergence and spread of antibiotic resistant bacteria. Resistant genes are often carried on plasmids and can spread quickly via horizontal gene transfer. Currently 90-95% of

Staphylococcus aureus strains are resistant to beta-lactam antibiotics. Opportunistic pathogens such as *S. aureus* are key species for developing antibiotic resistance. These pathogens are ubiquitous in nature and live symbiotically on or in humans. Hence, resistance genes are more easily acquired by these pathogens. After a major infection, transient colonies often remain to spread antibiotic resistance. Because of the growing concern over antibiotic resistance, many people have started looking for natural alternatives to antibiotics such as essential oils, honey and zinc oxide. I propose that chlorine dioxide (ClO_2) should be considered as an alternative to common antibiotics. ClO_2 disinfects via oxidation and is highly selective due to its one-electron transfer mechanisms and is currently used as a common disinfectant in: drinking water, dental practices, and as an aerosol. ClO_2 has even been effective in breaking down antibiotics that have built up in the environment such as: Tetracyclines, Fluoroquinolones and Beta-lactams.

Experimental Design

I will be measuring the susceptibility of *Enterococcus faecalis, Staphylococcus epidermidis* and *Bacillus subtilis* to penicillin and chlorine dioxide. The standard disk diffusion method will be applied using the minimum inhibitory concentrations of penicillin and chlorine dioxide on each disk (Andrew and Howe 2011). The minimum inhibitory concentration of penicillin has been found to be 10ug/disk (Andrew and Howe 2011). To obtain the minimum inhibitory concentration on chlorine dioxide, a phenol coefficient test will be completed (Reybrouck 1998). All tests will be conducted in triplicate for replication purposes.

The results will be evaluated based on the antibiotic susceptibility charts provided by CLSI and BSAC. Chlorine dioxide results will be evaluated by comparing its results with that of penicillin's. One can expect that chlorine dioxide will better inhibit all three pathogens in comparison to penicillin. This experiment will hopefully pave the way for eventual widespread use of chlorine dioxide as an internal antibiotic.

Methods

Part 1

A phenol coefficient test was conducted to determine the minimum inhibitory concentration of chlorine dioxide. Liquefied Phenol was obtained from Fisher Scientific (Cat # A931I-1). Phenol dilutions of 1:90, 1:95, 1:100, 1:105, and 1:110 were prepared in test tubes with water as a base and millilitres as units. Chlorine dioxide was obtained from Mountain Equipment Coop. (Product # 4013-495) made by Pristine Water Purification System. Chlorine dioxide dilutions of 1:300, 1:400, 1:500, 1:600, and 1:700 were prepared in test tubes with water as a base and millilitres as units. Typically, *Salmonella tyhpii* and *Staphylococcus aureus* are used for a standard phenol coefficient test; however, since these organisms were unavailable, *Staphylococcus epidermidis* was used instead. A suspension containing *S. epidermidis* was made and used to inoculate 20µl into all dilutions mentions above. One control test tube containing only water was also inoculated with 20µl of the bacterial suspension. All test tubes were put in a 37˚C water bath. After 5 minutes, 10 minutes, and 15 minutes, 1ml samples were taken from each test tube and inoculated into pre-labeled nutrient broth test tubes. Test tubes were temporarily taken out of the water bath and put in an ice bath during this time to ensure that all samples could be taken during intervals. Also, test tubes were agitated prior to inoculation as *S. epidermidis* is not motile. Inoculated test tubes were incubated in an agitating incubator at 37°C for 48hrs. Test tubes were evaluated based on whether there is growth or no growth in the tube. Growth was defined as whether the broth was cloudy (growth) or not (no growth). To ensure accuracy, all broth tubes were compared to the controls (which will show growth) and a broth test tube that was not inoculated (which will show no growth). The phenol dilution that allowed growth after 5 minutes in the water bath, but did not allow growth after 10 minutes or 15 minutes in the water bath, was the dilution used for comparison. Similarity, the chlorine dioxide dilution that allowed growth after 5 minutes in the water bath, but did not allow growth after 10 minutes or 15 minutes in the water bath, was the

minimum inhibitory concentration (MIC) used in part two. The phenol coefficient was calculated by dividing the reciprocal of the minimum inhibitory concentration found by the reciprocal of the phenol dilution found to have the same effect. If none of the chlorine dioxide dilutions above suited the criteria for MIC, new dilutions were picked based on analyzing the data and the test was run again.

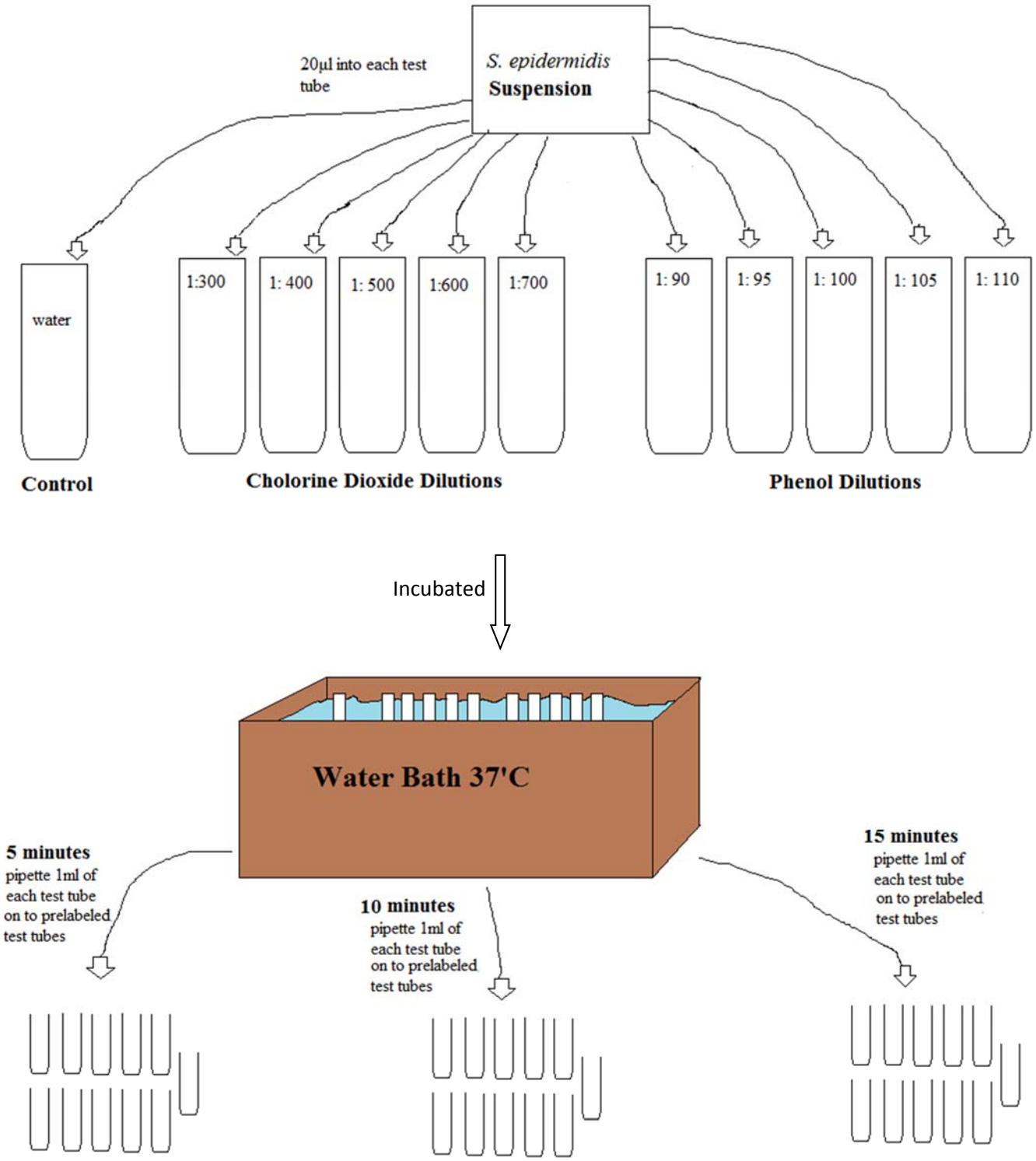

Figure 1: Illustration of Phenol Coefficient Test

Agent	Dilution	5 Minutes	10 Minutes	15 Minutes
Chlorine Dioxide	1:300			
	1:400			
	1:500			
	1:600			
	1:700			
Phenol	1:90			
	1:95			
	1:100			
	1:105			
	1:110			
Control	N/A			

Legend:

G= Growth
N= No Growth

Figure 2: Table Used to Display Part One Results

Part 2

The standard disk diffusion method was then carried out. A metal loop was sterilized and used to plate *Enterococcus faecalis* on to six TSA agar plates. A MIC penicillin disk was then placed on three of these plates. The other three plates received a disk that had been soaked in the MIC of chlorine dioxide. Plates were then labeled. The above method was repeated using *Staphylococcus epidermidis* and then again using *Bacillus subtilis.* At this point a blind testing protocol was conducted in which labels were removed from the plates and replaced with a numbered or color coded system not known by the experimenter. Plates were incubated for 24hrs at 37˚C. Plates

were taken out after 24hrs and the zone of inhibition was measured. The zone of inhibition was defined as the diameter of the circle where no bacteria grew. If a zone of inhibition was irregularity shaped the smallest diameter and the largest diameter were measured and the average was taken. The average zone of inhibition was calculated and recorded for each set of replicates. The numbered or color coded system was then decoded and results were interpreted.

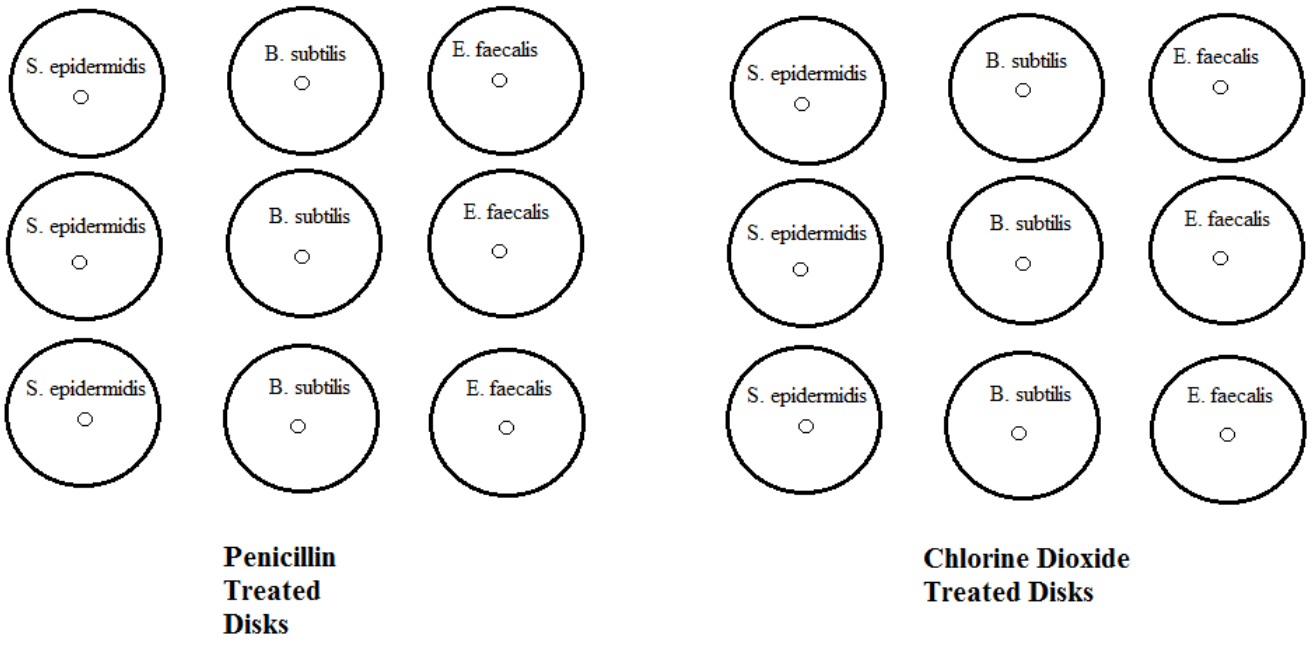

Figure 2: Illustration of Disk Diffusion Test

Agent	Replication Number	*Staphylococcus epidermidis*	*Bacillus subtilis*	*Enterococcus Faecalis*
Chlorine Dioxide	**1**			
	2			
	3			
	average			
Penicillin	**1**			
	2			
	3			
	average			

Figure 3: Table Used to Display Part Two Results

Budget

Below is a summary of my budget. For a more detailed breakdown on the budget please refer to the following page. I will be requiring a total of $200.19 to complete my research. Please note enough supplies are listed to complete part 1 three times and part 2 two times. This is to account for preliminary test runs of the experiment.

Product	**Price**
Supplies	$189.79
Equipment	Provided by Concordia
Lab Space	Provided by Concordia
Labour	Volunteer
Disposal of Waste	$0.40
Shipping	$10.00
Total	**$200.19**

Fig 4. Summary of Budget

Detailed Budget

Product	**Price of amount needed**	**Sigma aldrich order code**	**amount needed**	**amount bought**
Supplies				
Staphylococcus epidermidis	Provided by Concordia			
Bacillus subtilis	Provided by Concordia			
Enterococcus faecalis	Provided by Concordia			
Escherichia coli	Provided by Concordia			
Penicillin G	10.00	P3032-10MU	180ug	10mu
Chlorine dioxide	20.00	obtained from local store (mountain equipment coop)	about 40ml	60ml
Prepared Nutrient Agar	81.90	from hometrainingtools.com	135 plates	14 125ml bottles
Petri Dishes	40.10	from hometrainingtools.com	135 plates	140 plates
Pipette tips	11.22	Z637785-500EA	about 100	500
Sterile 10mm disks	19.87	74146-25DISCS-F	36	50
Phenol	2.50	P1037-25G	30mg	25g
Test tubes	4.20	T1536-250EA	about 30	250
Equipment				
Inoculating loops	Provided by Concordia			
500ml beaker	Provided by Concordia			
Adjustable volume pipette	Provided by Concordia			
Incubator	Provided by Concordia			
Forceps	Provided by Concordia			
Bench space	Provided by Concordia			
Hot water bath	Provided by Concordia			
Test tube rack	Provided by Concordia			
Labour				
Nadia Allen principal researcher	Volunteer			
Disposal of waste				
Disposable autoclave bag	0.40			
Shipping				
Sigma Aldrich	10.00			
Total	**200.19**			

Part One Results and Discussion

Part one trial one was conducted November 6-16, 2012 (Results available upon request). Plates were used instead of broth for this trial and test tubes were not agitated prior to sampling. Unfortunately all chlorine dioxide dilutions produced a lawn (1:300, 1:400, 1:500, 1:600, and 1:700). Because of these results, higher concentrations of ClO_2 are used in part one trial two, namely: 1:100, 1:200, 1:300, and 1:400. The pattern of growth seen on the phenol plates was confusing and led the experimenter to conclude that the plates were contaminated. The 1:105 phenol dilution produced the MIC pattern (growth at 5 minutes but not at 10 or 15 minutes) however, all of the higher concentrations of phenol produced a lawn. For these reason results of part one trial one are not included.

Results for part one trial two are recorded in table 1 below.

Agent	Dilution	5 Minutes	10 Minutes	15 Minutes
Chlorine Dioxide	1:100	G	N	G
	1:200	G	G	G
	1:300	G	G	G
	1:400	G	G	G
Phenol	1:80	N	N	N
	1:85	N	N	N
	1:90	G	N	N
	1:100	G	N	N
Control	N/A	G	G	G

Legend:
G= growth
N= no growth

Table 1: Part one trial two results. Points of interest are highlighted in red.

In the table above both the phenol dilutions of 1:90 and 1:100 show the MIC pattern. The 1:80 and 1:85 phenol dilutions did not grow at any of the three times which further points to 1:90 and 1:100 being the MIC of phenol. Because 1:100 is the lowest concentration with the MIC pattern it will be used as my MIC for phenol. It is possible that lower concentrations of phenol such as 1:105 or 1:110 also show a MIC pattern but for the purposes of this experiment and due to lack of time 1:100 will be used as the MIC of phenol and phenol dilutions will not be repeated for trial three or four of this experiment.

All of the ClO_2 tubes in part one trial two grew except the 1:100 dilution at 10 minutes. This is discouraging as the experimenter was lead to believe that the MIC of ClO_2 is much lower than these results show. Stampi and others (2002) found that *Escherichia coli* count could be reduced 99.97% with the usage of 2.2mg/L of ClO_2, which amounts to a dilution of 1:450. Upon further analysis though, Stampi and others (2002) allowed the *E. coli* to interact with the ClO_2 for 20 minutes and hence obviously did not use the MIC. As mentioned above 1:100 dilution at 10 minutes did not grow. These results were confusing at first however part one trial three and four shown in table 2 below sheds light on this strange pattern.

ClO_2 Dilutions	**5 Minutes**	**10 Minutes**	**15 Minutes**
1:50	N	N	N
1:67	N	N	N
1:75*	N	N	N
1:100	G	N**	N
CONTROL	G	G	G

Legend:
G= growth
N= no growth

Table 2: Part one Trial three and four results. Points of interest are highlighted in red.

*Originally in trial 3 the 1:75 dilutions all grew. Upon repeating the 1:75 dilution in trial 4, none of the dilutions grew. It is suspected that the 1:75 dilution for trial 3 was contaminated. Therefore the trial 3 results for 1:75 were discarded and the results show in table 2 for 1:75 dilutions are from trial 4.

** This result it exclusively from trial three, upon repeating the 1:100 dilution in trial four, the 1:100 dilution at 10 minutes grew.

As the 1:50 to 1:75 dilutions in table 2 show, ClO_2 does have an inhibition effect on bacteria as many researchers such as Rajkovic and others (2009) and Stampi and others (2002) report. These results are also consistent with Grootveld and others (2001) study where a 1:50 dilution with a contact time of just 3 minutes was used to prove ClO_2 effectiveness as a disinfectant.

The 1:100 dilution in table 2 shows the MIC pattern for ClO_2. It is believed that 1:100 is on the very edge of the MIC range for ClO_2 because, in both trials two and four, this dilution showed strange patterns of inhibition. In trial two, seen in table 1, the 1:100 dilution grew at both 5 minutes and 15 minutes but not at 10 minutes. In trial four the 1:100 dilution grew at both 5 minutes and 10 minutes but not at 15 minutes. Further trials could not be conducted for dilutions between 1:75 and 1:100 due to lack of time, however if further trials were conducted they would likely show a more accurate MIC for ClO_2. For the purpose of this experiment 1:100 will be used at the MIC of ClO_2.

Because 1:100 is the MIC found for both ClO_2 and Phenol, the phenol coefficient is 1. This indicated that ClO_2 is as effective as phenol in inhibiting bacterial growth. Though ClO_2 was found to be less effective than the experimenter was lead to believe by research, it is noteworthy that ClO_2 is on par with the standard in which all disinfectants are measured against.

Part Two Results and Discussion

The results for part two trial one are recorded in table 3 below.

Agent	**Replication Number**	*Staphlyococcus epidermidis*	*Bacillus subtilis*	*Enterococcus faecalis*
Chlorine Dioxide	1	0	0	0
	2	0	0	0
	3	0	12	0
	Average*	0mm	4mm	0mm
Penicillin	1	17	0	18
	2	17	19	17
	3	14	0	17
	Average*	16mm	6mm	17mm

Table 3: Part two trial one results. Zone of Inhibition Diameters (in millimetres) for Chlorine Dioxide and Penicillin in relation to *Staphlyococcus epidermidis, Bacillus subtilis,* and *Enterococcus faecalis*.

* Averages rounded to the nearest millimetre, 0.5 is rounded up.

The results in table 3 show that ClO_2 was very ineffective in inhibiting *Staphlyococcus epidermidis, Bacillus subtilis*, and *Enterococcus faecalis* at the MIC found. The only ClO_2 treated plate that showed a zone of inhibition was replicate 3 of *B. subtilis*. This would normally be counted as contamination, however a similar pattern of inhibition is seen in the penicillin treated *B. subtilis* plates. The penicillin replicate 2 for *B. subtilis* showed a zone of inhibition but replicates 1 and 3 did not have a measureable zone of inhibition. Further, there is no zone diameter interpretation charts containing *Bacillus spp.* for penicillin. According to page 27 of the M45-P document of the Clinical and Laboratory Standards Institute (CLSI) (2005) "Recommendations for disk diffusion of *Bacillus spp.* cannot be made as limited data exists for disk diffusion testing of this genus". This statement is somewhat confusing as why would limited data exist for such a common genus? This researcher believes perhaps *Bacillus spp.* behaves unpredictably in disk diffusion tests and a zone of inhibition range is

difficult to pin down for this genus. These results would definitely support such a hypothesis, however further testing would need to be conducted to confirm it.

Due to the disappointing results found for ClO_2 at the MIC, a second trial was conducted with ClO_2 dilutions of 1:50 and 1:75. The results of this trial are shown in table 4 below.

Dilution of ClO_2	**Replication Number**	*Staphlyococcus epidermidis*	*Bacillus subtilis*	*Enterococcus faecalis*
1:50	1	0	0	0
	2	0	0	0
	3	0	0	0
	Average	0mm	0mm	0mm
1:75	1	0	0	0
	2	0	0	0
	3	0	0	0
	Average	0mm	0mm	0mm

Table 4: Part Two Trial Two Results. Zone of Inhibition Diameters (in millimetres) for Chlorine Dioxide Dilutions in Relation to *Staphlyococcus epidermidis, Bacillus subtilis,* and *Enterococcus faecalis*.

Table 4 shows that both the 1:50 dilution and the 1:75 dilution failed to show any zone of inhibition on any of the bacteria. Since we know from part one of the experiment that 1:50 and 1:75 dilutions have an inhibitory effect on *S. epidermidis,* this data leads the experimenter to suspect a flawed experimental design and that the discouraging results found for the ClO_2 MIC might be misleading.

It is possible that the ClO_2 disk dried rapidly in the incubator and once dry, the inhibitory effects of the ClO_2 were significantly reduced. This reduction in effectiveness would allow the bacteria to sweep in and grow around the disk. Perhaps if the plates were checks after only 12 hours, a zone of inhibition would be seen.

It is also possible that the MIC of ClO_2 changes with the type of bacteria it is inhibiting. Meneghin and others (2008) tested the effect of ClO_2 on several alcohol fermenting bacteria including *Bacillus subtilis, Leuconostoc mesenteroides* and many *Lactobacillus* species. Like the results from this experiment, The MIC of

ClO_2 on *Bacillus subtilis* was found to be 1:100, however, the MIC for each organism was different and ranged from a 1:100 dilution to a mere 1:8 dilution (*Lactobacillus plantarum*). The findings of Meneghin and others (2008) suggest that perhaps the MIC for all three opportunistic pathogens should have been found prior to completion of part two of the experiment. The findings that *B. subtilis* has a known ClO_2 MIC of 1:100 may explain the one *B. subtilis* ClO_2 1:100 plate that had a noticeable zone of inhibition, but it does not explain why the 1:50 and 1:75 ClO_2 plates for *B. subtilis* had no zones of inhibition. The fact that the data from part one trial two does not line up with the known MIC of ClO_2 for *B. subtilis* leads the experimenter to further conclude that there is something wrong with the experimental design.

According to the zone diameter interpretation charts from CLSI and British Society for Antimicrobial Chemotherapy (BSAC) the penicillin used was also ineffective at inhibiting two of the three bacteria namely *Staphlyococcus epidermidis,* and *Bacillus subtilis*. Though there is no zone diameter interpretation chart for Bacillus spp., as mentioned above, the M45-P document of the CLSI (2005) does recommend the use of the zone diameter interpretation chart for Staphlyococcus spp. to interpret Bacillus spp. data. The zone diameter interpretation chart for Staphlyococcus spp. states that the zone of inhibition for penicillin must be above 28mm to be considered susceptible. In table 3 the average penicillin zone of inhibition for *S. epidermidis* is 16mm and the average penicillin zone of inhibition for *B. subtilis* was 6mm. this data shows that the *S. epidermidis,* and *B. subtilis* strains used are resistant to penicillin.

The zone diameter interpretation chart for Enterococcus spp. on penicillin states that the zone of inhibition diameter must be above 14mm to be considered susceptible. Table 3 shows that the average penicillin zone of inhibition for *Enterococcus faecalis* is 17mm and hence this strain of *E. faecalis* is susceptible to penicillin.

It should be noted that, due to lack of supplies, the penicillin used expired in September 2010. This could have changed the results found. It is possible that expired penicillin is less effective that current penicillin and could explain why two of the three opportunistic pathogens were found to be resistant to penicillin.

However, resistance to penicillin is common in the microbial world so the two penicillin resistant pathogens found in this experiment is not a point of concern.

It should also be noted that the blind testing protocol did not go as planned. Because the penicillin disks were manufactured elsewhere, they each had a small label printed on them. This label allowed the experimenter to tell the difference between the penicillin treated plates and the chlorine dioxide treated plates. The experimenter could not tell the difference between the three pathogens however, so this afforded some level of blind testing. Still, if a true blind testing protocol is to be conducted in the future, the researcher suggests that 10ug of penicillin be manually inoculated onto blank disks that are identical to the disks soaked in the MIC of chlorine dioxide.

Conclusion

The MIC of chlorine dioxide was found to be 1:100 dilution. This concentration is on par with the MIC found for phenol and indicates that chlorine dioxide is as effective as phenol in inhibiting bacterial growth. However, when the MIC found for ClO_2 was put to the test against three opportunistic pathogens, only one plate out of nine showed a visible zone of inhibition. Compared to penicillin, ClO_2 was found to be less effective against the three opportunistic pathogens tested, namely: *Staphlyococcus epidermidis, Bacillus subtilis*, and *Enterococcus faecalis*. These results may in fact be erroneous however as part two trial two of the experiment shows evidence for a flawed experimental design. It is recommended by this researcher that further avenues for testing and comparing the inhibitory effect of ClO_2 to that of penicillin be explored.

Though penicillin did produce a visible zone of inhibition for seven of the nine plates, according to zone interpretation charts, *S. epidermidis* and *B. subtilis* were found to be resistant to the penicillin used. It is recommended by this researcher that future research be conducted on the effectiveness of expired penicillin versus new penicillin. Future research could also be conducted on the genus Bacillus spp. and why its performance in the disk diffusion method is unpredictable.

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