Title
Inactivation and Reactivation of Ampicillin-Resistant Escherichia coli K12 Due to Chlorination

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Inactivation and Reactivation of Ampicillin-Resistant *Escherichia coli* K12 Due to Chlorination

A thesis submitted in partial fulfillment
of the requirements for the degree of Master of Science
in Civil Engineering

by

Hyosun Kim

2016

ABSTRACT OF THE THESIS

Inactivation and Reactivation of
Ampicillin-Resistant *Escherichia coli* K12 Due to Chlorination

by

Hyosun Kim

Master of Science in Civil Engineering
University of California, Los Angeles, 2016
Professor Jennifer A. Jay, Chair

Due to the growing concern about antibiotic-resistant bacteria (ARB) in water, there have been many studies on inactivation of ARB by chlorination. In order to examine possible increase in ARB concentrations after a retention time, this study was performed to explore not only inactivation, but also reactivation of *Escherichia coli* (*E. coli*) K12. Observed immediately after chlorination for 10 minutes, inactivation results demonstrated that even ampicillin concentration of 2 mg/L enhances inactivation of bacteria nearly up to 100%. Reactivation of surviving cells was observed after 24-h retention time in the study. Reactivation results showed that MIC_{50} and MIC_{90} for 0 mg Cl_2/L were below 4 mg/L of ampicillin. However, both MIC_{50} and MIC_{90} were much higher after chlorination; MIC_{50} and MIC_{90} for 1 mg Cl_2/L were greater than 16 mg/L of ampicillin. The study indicates that there is a risk of regrowth of ARB even after 1-day of standing period. Therefore, immediate attention on ARB treatment is required to protect public health.

The thesis of Hyosun Kim is approved.

Shaily Mahendra

Michael K. Stenstrom

Jennifer A. Jay, Committee Chair

University of California, Los Angeles

2016

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Finally, I would like to thank my family who was always there supporting and encouraging me.

CHAPTER I: INTRODUCTION

In order to reduce waterborne diseases to protect public health, chlorine was first used as a disinfectant for drinking water systems in the U.S. in 1908 (USEPA, 2000). The U.S. Public Health Services then implemented standards such as 1962 U.S. Public Health Service Standards in order to regulate contaminants of growing concerns, such as arsenic and lead. However, many water supplies still did not meet the U.S. Public Health standards. Environment and health concerns eventually led to the development of environmental and health laws, which include Safe Drinking Water Act of 1974. With the 1986 amendment, the U.S. Environmental Protection Agency (EPA) administered standards and made changes to disinfection methods to regulate contaminants and disinfection by-products (DBPs) (Dyksen et al., 2007; Levin et al., 2002). In order to provide a certain level of water quality and protect public health, the U.S. EPA has set a Maximum Residual Disinfectant Level (MRDL) for chlorine, which is the maximum limit of chlorine allowed in drinking water, as 4 mg/L and the minimum chlorine residual concentration as 0.2 mg/L at point-of-entry (POE) (Lantagne, 2008).

Disinfection is a process that inactivates harmful microorganisms such as *Giardia*, *Cryptosporidium*, and some viruses, in water and wastewater treatment facilities. There are several different methods of disinfection and one of the most common methods is chlorination. Chlorination is widely used for water and wastewater treatment plant effluents because its application is simple and fairly inexpensive (Huang et al., 2011; USEPA, 2013). However, many studies demonstrated that chlorination creates DBPs and is not as effective for removing certain bacteria and contaminants, including antibiotic-resistant bacteria.

Utilizing the bacterial growth inhibition mechanisms of antibiotics, humans have been producing antibiotics in order to treat the infections in humans, agriculture, and animals/fish. The increased availability of antibiotics substantially raised human's intake as well as excretion of antibiotics into the waste stream over a period of time. Therefore, microorganisms are readily exposed to antibiotics and the exposure triggered antibiotic resistance mechanisms of microorganisms. Likewise, ARB are increasing in frequency in aqueous environments, such as potable water and wastewater treatment plants, and they are considered as waterborne contaminants that threaten the public health (Pruden et al., 2006; Huang et al., 2011).

Several studies have looked at inactivation of ARB after chlorination and only a couple of studies have looked at reactivation of ARB after the retention time. Huang et al. (2011), for example, has shown reactivation results that indicated ARB concentrations increased considerably at low chlorine concentration. In order to investigate further risk of ARB regrowth in water storage or distribution system and the environment, this study of both inactivation and reactivation of ARB was performed.

The primary goal of this thesis is to find out the potential risk of ampicillin resistance for *E. coli* K12 after chlorination. The specific objectives of this thesis include:

- To evaluate the inactivation of ampicillin-resistant bacteria immediately after chlorination
- To evaluate the reactivation of ampicillin-resistant bacteria after chlorination with 24-hour standing period

Null hypothesis of this thesis is that chlorination does not affect the selection of ampicillin-resistant bacteria and alternative hypothesis is that chlorination increases the ampicillin resistance of surviving bacteria.

CHAPTER II: LITERATURE REVIEW

The purpose of this chapter is first to provide basic definitions and mechanisms of processes used in the study. With the basic concepts introduced, several studies on the effects of chlorination on antibiotic-resistant bacteria are presented.

2.1 Chlorine and Chlorine Resistance

Chlorine is an oxidizing agent that can be added into water in either gaseous or aqueous form as a disinfectant. For example, sodium hypochlorite (NaOCl), which is one of the aqueous solutions used in the study, hydrolyzes as the following reaction:

$$NaOCl + H_2O \rightarrow Na^+ + HOCl + OH^-$$

Then, hypochlorous acid (HOCl) disassociates as the following:

$$HOCl \rightleftharpoons H^+ + OCl^-$$

This reaction is incomplete in water, which has a neutral pH. Therefore, both hypochlorous acid and hypochlorite ions (OCl^-) will be present in such water and they are referred to as free available chlorine (FAC). When free available chlorine reacts with ammonia, which is commonly present in water and wastewater, several other forms such as monochloramine, dichloramine and trichloramine are produced. These chloramines are referred to as combined available chlorine (Harp, 2002; USEPA, 2013). FAC, especially HOCl, is more effective oxidant and disinfectant than combined available chlorine because it can react quickly with many compounds. Because of its reactive tendency, it is suitable as a primary disinfectant but it is unstable as a residual. In contrast, chlorine derivatives such as monochloramines are less reactive so they are more stable and long lasting as a residual (USEPA, 2013). They can diffuse

into the cell, modify bases in DNA, and cause mutagenesis within microorganisms to damage DNA (Thomas et al., 1987; Dukan and Touati, 1996).

Chlorine is a disinfectant because it kills microorganisms by penetrating the cell wall and cell membrane of microorganisms. It affects the cell permeability and the protein synthesis which impedes many transport processes such as amino acid transport. It eventually damages the DNA of microorganisms and causes inactivation (Haas & Engelbrecht 1980; Dukan and Touati 1996; Rosenkranz, 1973). In order to calculate the inactivation ratio by chlorination, chlorine concentration (C) in mg/L and corresponding contact time (T) in minutes before any chlorine application point must be determined. CT value represents the product of C and T and it refers to chlorine dosage required to disinfect water.

Bactericidal chlorination, however, still can be resisted by bacteria. Shuval et al. (1973) showed that coliforms in chlorinated samples reactivated after holding period even though the number of coliforms was reduced or not detected immediately after chlorination. This laboratory study demonstrated that even though chlorine residual was not neutralized, the exposure to initial chlorine dose up to 11 mg/L caused regrowth of coliforms over the period of retention time and the field study showed that regrowth of coliforms decreased as the residual chlorine in the storage was increased. Likewise, both the field and laboratory experiments in this study showed that regrowth of coliforms occurred in chlorinated samples unless certain concentration level of chlorine was achieved. Li et al. (2013) investigated the potential selection of pathogenic bacteria that allow themselves to survive and reproduce under the environmental pressure due to chlorination. His results demonstrated that low chlorine dose is likely to induce reactivation of bacteria and thus, chlorination influences the selection of chlorine-resistant bacteria.

Microorganisms resist to each stress they encounter by inducing specific proteins (Gottesman 1984; Jenkins et al. 1988). After exposure to any environmental stresses such as starvation due to low-nutrient solution, they can be more resistant to oxidation such as free chlorine because the enhanced resistance can be associated with starvation protein synthesis (LeChevallier et al., 1988; Jenkins et al., 1988). DNA-binding protein from starved cells (Dps) is synthesized in *E. coli* by stresses such as the cessation of growth and starvation, and it protects the DNA from oxidation and regulates gene expression (Almiron et al.,1992). Likewise, the regrowth of survivors that resisted stresses is likely due to the absence of competitive microorganisms (Shuval et al., 1973), the change of DNA structure of microorganisms by oxidative species, and the induction of protective functions of DNA-binding proteins (Rosenkranz, 1973). These factors, after all, may cause further difficulty in treatment.

2.2 DPD Colorimetric Method

The N, N-diethyl-p-phenylenediamine (DPD) colorimetric method determines free or total residual chlorine in water and wastewater. When chlorine is present in water at a pH 7, it oxidizes the DPD amine and produces two oxidation products (Figure 2.1). The main oxidation product, which is the Wurster dye, is a free radical species that turns the water sample into a magenta color if chlorine is present in water. This dye color is then measured with a spectrophotometer at a wavelength between 490 and 555 nanometers (nm) (Harp, 2002). After reading the intensity of the color, the spectrophotometer displays the measurement of chlorine residual concentration in mg/L.

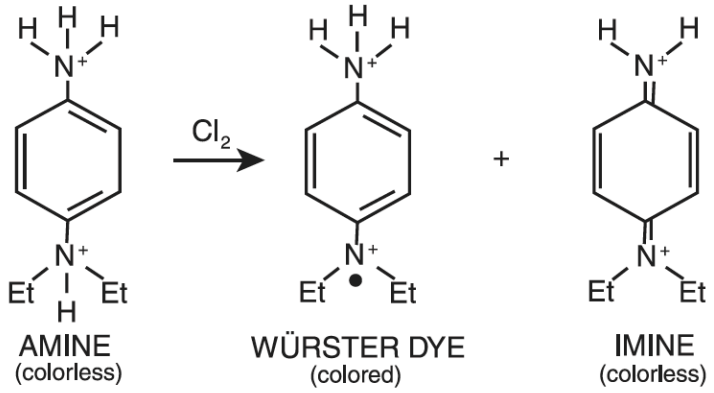

Figure 2.1. DPD-chlorine reaction (Harp, 2002)

2.3 Antibiotics and Antibiotic Resistance

Many microorganisms in the environment are capable of producing antibiotics. The function of their antibiotics is to inhibit the growth of other organisms. In order to protect themselves, these antibiotic-producing microorganisms have determinants to resist their own antibiotics. Bacteria that consume those antibiotics as nutrients, however, also have a capability to resist those antibiotics due to their intrinsic and extrinsic resistance mechanisms that help bacteria survive (Martínez, 2008). The mechanisms of antibiotic resistance include the following: inhibition of antibiotics' access into target by reducing permeability and increasing multidrug resistance (MDR) efflux pumps activity, changes in the target structure by mutation or simple protection, and modification of antibiotics (Blair et al. 2015).

The minimum inhibitory concentration is the lowest concentration of an antibiotic that inhibits the growth of bacteria. The concentration of antibiotics that inhibit the bacteria population by 50% is called hemi-inhibitory concentration (MIC_{50}) and 90% is called MIC_{90} (Pang et al., 2015; Bulus et al., 2016).

Ampicillin is classified as the penicillin group of bactericidal β-lactam antibiotics that trigger competitive inhibition of penicillin-binding proteins in order to inhibit cell wall synthesis

or disrupt the cell (Malouin and Bryan, 1986; Templeton et al., 2009). Likewise, ampicillin prevents cross-linkages of the outside layer of membrane of bacteria, called peptidoglycan as shown in Figure 2.2 (Malouin and Bryan, 1986; Huang et al., 2011). The mechanisms of ampicillin resistance include bacterial enzyme β-lactamases destroying ampicillin or modifying the binding proteins (Neu, 1984; Malouin and Bryan, 1986; Huang et al., 2011). There were several studies on antibiotic resistance. Reinthaler et al. (2002), for example, showed that resistance rate for *E. coli* against ampicillin was between 2 % and 18 %.

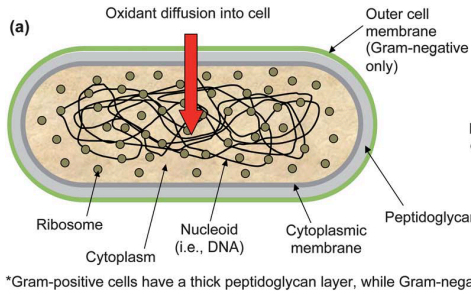

Figure 2.2. Illustration of a bacterial cell structure (Dodd, 2012)

The spread of antibiotic resistance may occur by vertical transfer, such as cell division, and horizontal gene transfer, including conjugation, transduction, and natural transformation (Figure 2.3). Conjugation is a process whereby an exchange of genes between active donor and viable recipient bacterial cells takes place by physical contact. Transduction is a process in which DNA packaged in bacteriophage particles is transferred from infected bacterial donor cells to recipient cells after cell lysis or death. Natural transformation is a process whereby DNA from a donor cell is transported into competent recipient cells (Dodd, 2012). Due to these gene transfer processes of microorganism, the population of ARB can increase tremendously by simple introduction of ARB to the environment.

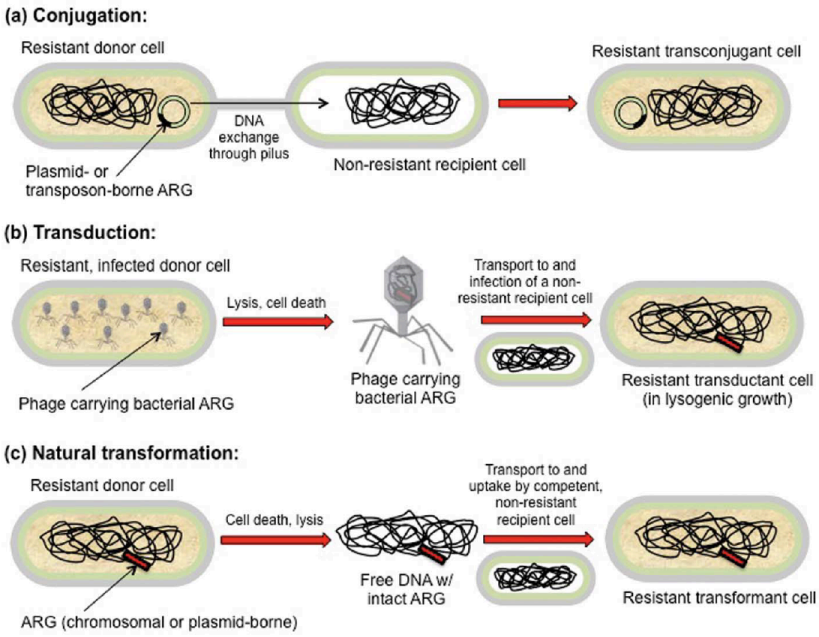

Figure 2.3. Illustrations of horizontal gene transfer mechanisms: (a) conjugation, (b) transduction, and (c) natural transformation (Dodd, 2012)

2.4 Antibiotic Resistance to Chlorination

The increased use of antibiotics has escalated the risk of antibiotic resistance in water and wastewater (Rizzo et al., 2013). Few studies have shown that chlorination may not cause the selection of ARB. For example, Templeton et al. (2009) looked at inactivation of chlorinated ampicillin-resistant *E. coli*. The experiment by Templeton et al. (2009) was completed by exposing the samples to ampicillin first and then chlorinating the samples. The results demonstrated that chlorination does not select antibiotic-resistant survivors. In contrast, several other studies have shown that chlorination at water and wastewater treatment plants may induce the proliferation of ARB.

Similar to Templeton et al. (2009), Pang et al. (2015) performed the experiment by exposing the samples to ampicillin first and then chlorinating. This paper observed the behavior of ampicillin resistance for *E. coli* after exposure to chlorine by looking at the change in the

ampicillin hemi-inhibitory concentration (MIC_{50}) before and after chlorination. Due to the increase in MIC_{50} after chlorination, the results showed that there is a potential increase in the risk of high ampicillin resistance for *E. coli*.

Huang et al. (2011) observed the inactivation and reactivation behavior of total heterotrophic bacteria and antibiotic-resistant bacteria after chlorination. These authors used the secondary effluent samples from a municipal wastewater treatment plant and their results showed the number of ampicillin-resistant bacteria decreased as the concentration of sodium hypochlorite increased. After standing period of 22-hour, the chlorine doses of 0.5 and 1.0 mg/L showed a significant increase in antibiotic-resistant bacteria.

Similar to Huang et al. (2011), the experiment for this thesis was completed by chlorinating the samples first and then exposing them to ampicillin to examine both inactivation and reactivation behavior of bacteria after chlorination. However, a specific bacteria strain was used in the study, whereas secondary effluent samples were used in Huang et al. (2011). Therefore, the study allowed the evaluation of the specific strain, *E. coli* K12, affected by chlorination and ampicillin.

CHAPTER III: EXPERIMENTAL METHODOLOGY

3.1 Experimental Procedure

3.1.1 Experiments

Three experiments were performed over three weeks. Each experiment was conducted with a control group, which was 0 mg/L of chlorine concentration and 0 mg/L of ampicillin concentration, and an experimental group, which was 1, 2, and 10 mg/L of chlorine and 4, 8, and 16 mg/L of ampicillin. First, second, and third experiment were completed with a control group and chlorine concentration of 1, 2, and 10 mg/L, respectively. Each experiment was comprised of four samples for control group (total culture samples, n = 4), four samples per chlorine concentration for 0 mg/L of ampicillin (total culture samples, n = 4), and two samples per chlorine concentration for each ampicillin concentration (total culture samples, n = 12).

To validate the results, another experiment was conducted with a control group, which was 0 mg/L of chlorine concentration and 0 mg/L of ampicillin, and an experimental group, which was 1 and 10 mg/L of chlorine concentrations and 2, 4, 16, and 32 mg/L of ampicillin concentrations. The experiment consisted of four samples for the control group (total culture samples, n = 4), four samples per chlorine concentration for 0 mg/L of ampicillin (total culture samples, n = 8), and two samples per chlorine concentration for each ampicillin concentration (total culture samples, n = 24).

3.1.2 *E. coli* K12 Sample Preparation

E. coli is Gram-negative bacteria that are sensitive to free chlorine and it is considered as a model organism for disinfection testing (Cherchi and Gu, 2011). Among *E. coli* strains, K12 strains are known as a laboratory model because it has several advantages: they are living organism that their genetics are well known, their genes can be easily modified, and they are biologically safe (Kuhnert and Frey, 1995). Because *E. coli* K12 strains are nonpathogenic, they were used for the study.

In order to obtain pure culture of *E. coli* K12, discrete colonies were isolated by performing a streak-plate. This pure colony contains identical copies of the original cell and it was used for the experiment. The first section of the plate was streaked with a sterile loop after the initial inoculation. Then, the first section was overlapped one time and the second section was streaked with the sterile loop again. The same procedure was repeated for the third section by overlapping only with the second section once and streaking in the third section of the plate. Subsequently, the plates were incubated at 37° C for 24-hour to let the colonies form. After the pure colonies were obtained, triplicate of *E. coli* samples were grown in Falcon centrifuge tubes containing nutrient broth (3.0 g/L beef extract, 5.0 g/L peptone) at 25 °C. The bacteria samples were transferred by serial 10-fold dilutions to new nutrient broth approximately every two or three days to keep them in exponential phase. They were transferred to new media the day before each experiment to ensure exponential phase.

3.1.3 Ampicillin

Ampicillin stock solution of 5000 mg/L concentration was prepared with deionized (DI) water and sterile filtration was completed using 20 mL syringe and 0.22 μm syringe filter. The

solution was stored in 1 mL aliquots in freezer at -20 °C. These aliquots were used on the day of making nutrient agar plates with specific ampicillin doses (0, 2, 4, 8, 16, and 32 mg/L).

3.1.4 Agar Plates Preparation

Nutrient agar containing in a ratio of 8 g of nutrient to 15 g of agar was sterilized at 121°C. After sterilization, nutrient agar was cooled down to a desirable temperature, which is at or below 55°C and specific ampicillin doses ranging from 0 to 32 mg/L (0, 2, 4, 16, 32 mg/L) were added. Each mixture was stirred with a magnetic stir bar and 20 mL of nutrient agar with ampicillin at specific doses were poured into each 90mm Petri dishes in duplicate.

3.1.5 Ammonia Washing

In order to remove ammonia that will react with chlorine, samples were washed with sterile phosphate-buffered saline (PBS) at pH 7.4 by centrifuging at 7,830 rpm at 4 °C three times for 15 minutes on the day of the experiment. After three washes, 5 mL of washed samples was pipetted into Hach Ammonia TNT Plus vials (Loveland, CO) and reacted for 15 minutes. Then, the vials were measured for the ammonia residual using spectrophotometer. The concentration of suspended *E. coli* K12 was then measured using spectrophotometer and the samples were transferred into sterile 250 mL Erlenmeyer flasks to maintain 10^7 colony forming units (CFU)/mL as the initial concentration of *E. coli* K12 for each experiment.

3.1.6 Chlorination

The concentration of chlorine stock solution was first checked with DPD colorimetric method. Since phosphate buffer does not produce toxic derivatives of HOCl, phosphate buffer saline (PBS) was used in the study (Dukan and Touati, 1996).

After washed samples of *E. coli* K12 were transferred into sterile 250 mL Erlenmeyer flasks with magnetic stir bars at room temperature (25 °C), chlorine concentrations were added using the stock solution. After 10 minutes of contact time, chlorine concentrations were measured using Hach DPD Free Chlorine Powder Pillow to determine the chlorine demand of *E. coli*. Then, a sodium thiosulfate solution (1.5%) was added to terminate the chlorination.

Program 88 Chlorine F&T HR for chlorine concentration ranging from 0.1 to 10 mg/L was started on spectrophotometer. A sample cell was filled up to the 5-mL mark and the blank sample cell was cleaned with Kimwipes. After inserting the blank into the cell holder, ZERO button was pressed to set 0.0 mg/L Cl_2. Then, one Hach DPD Free Chlorine Powder Pillow was added into the sample cell. After letting the reaction for about 20 seconds, the sample cell was cleaned with Kimwipes. Then, the sample was inserted into the spectrophotometer in order to measure the chlorine residual.

3.1.7 Plating

In order to obtain between 30 and 300 CFU per plate, 10-fold serial dilutions for samples were completed using PBS and in duplicate. 50μL of each sample was plated on 90mm Petri dishes containing the nutrient agar with specific ampicillin concentrations, which were prepared in advance. After plating, the plates were incubated at 37 °C for 24 hours. On the following day,

another set of 10-fold serial dilutions for samples was completed again using PBS for the reactivation purpose. The plates were then again incubated at 37 °C for 24 hours.

3.1.8 Log Inactivation and Percentage of ARB Equations

Huang et al. (2011) used the following equations and notations to examine the log ratio of inactivation of specific bacteria by chlorination and the percentage of antibiotic-resistant bacteria, respectively:

$$\text{Inactivation of specific bacteria j} = \log\left(\frac{N_0^j}{N_i^j}\right) \qquad (1)$$

N_0^j: plate count of the specific bacteria j, before chlorination (CFU/mL)

N_i^j: immediate survival of the specific bacteria j after chlorination at a chlorine dosage of *i* (CFU/mL)

$$\text{Percentage of antibiotic} - \text{resistant bacteria } (\%) = \frac{N_i^j}{N_i^0} \times 100\% \qquad (2)$$

N_i^j: immediate survival of the specific bacteria j after chlorination at a chlorine dosage of i (CFU/mL)

N_i^0: immediate survival of bacteria with 0 mg/L of ampicillin after chlorination (CFU/mL)

i = 0, when the dosage of chlorination was 0.

CHAPTER IV: RESULTS AND DISCUSSION

4.1 Results

4.1.1 Inactivation

Inactivation of *E. coli* K12 by chlorination was investigated immediately after chlorination. Figure 4.1 illustrates the effectiveness of chlorine on inactivating *E. coli*. Chlorinated samples with 0 mg/L of ampicillin resulted in distinctive increase in inactivation due to chlorination. A 1 mg/L of chlorine concentration inactivated *E. coli* by 3-log and a 10 mg/L of chlorine concentration inactivated *E. coli* by 4-log.

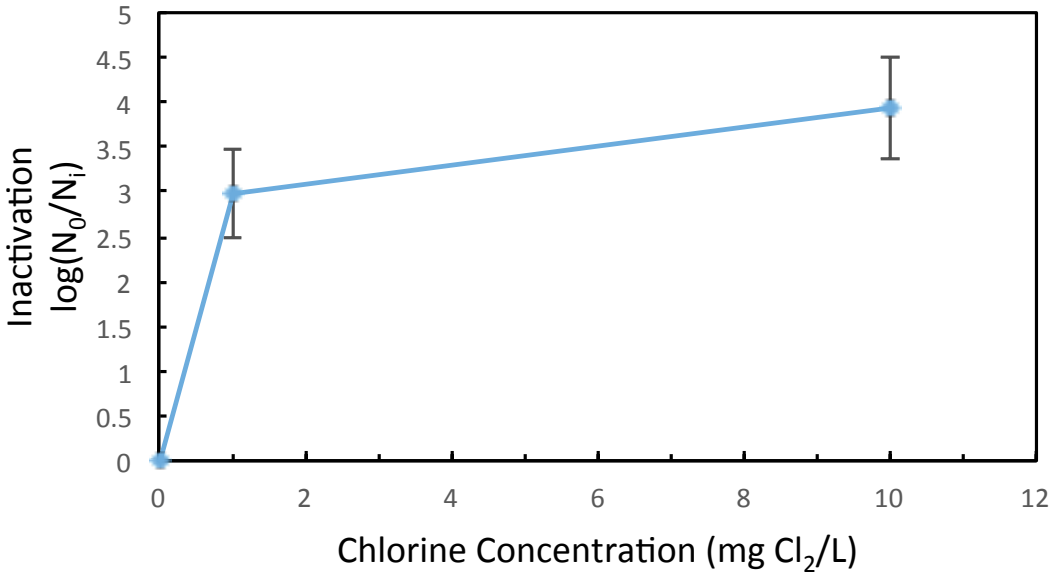

Figure 4.1. Inactivation rate of average *E. coli* concentrations of four replicates immediately after chlorination for 0 mg/L of ampicillin. The contact time of chlorination was 10 min.

When ampicillin was introduced to samples that were chlorinated with 0, 1, 2 and 10 mg Cl_2/L, the number of *E. coli* colony counts had been reduced to 0 or nearly 0 by chlorination. These counts were far below the preferred range from 30 to 300 colonies. Therefore, log inactivation after the exposure to ampicillin could not be calculated. After all four experiments, it

can be inferred that the combination of chlorine and ampicillin inactivated *E. coli* K12 nearly 100 % and a 2 mg/L of ampicillin concentration was sufficient to inactivate *E. coli*.

4.1.2 Reactivation

Reactivation of ampicillin-resistant bacteria was observed after leaving samples that were chlorinated with 0, 1, 2 and 10 mg Cl_2/L in the dark for 24 hours then exposing them to ampicillin concentrations of 0, 4, 8, and 16 mg/L. Concentrations of viable cells after a standing period of 24-h are shown in Figure 4.2, Figure 4.3, and Figure 4.4. These concentrations may be due to regrowth of living bacteria, reactivation of inactivated bacteria by chlorination, and regrowth of reactivated bacteria (Huang et al., 2011). MIC_{50} and MIC_{90} are ampicillin concentrations that inhibited 50% and 90% of viable bacteria concentrations at 0 mg/L of ampicillin and they are summarized in Table 4.1. These MIC values show a shift in ampicillin resistance before and after chlorination.

Results showed that 4 mg/L of ampicillin was sufficient to reduce number of *E. coli* colonies chlorinated with 0 mg Cl_2/L by 90% of the control concentration; this ampicillin concentration was equivalent to MIC_{90} for unchlorinated samples (Figure 4.2, Figure 4.3, and Figure 4.4). As shown in Figure 4.2, ampicillin-resistant bacteria after 1 mg/L of chlorination reactivated tremendously. Thus, both MIC_{50} and MIC_{90} for 1 mg Cl_2/L of chlorine must be higher than 16 mg/L of ampicillin dose in order to inhibit 50% and 90% of the bacterial concentration after 1 mg Cl_2/L of chlorination and 0 mg/L of ampicillin exposure. As shown in Figure 4.3, MIC_{50} and MIC_{90} for 2 mg Cl_2/L were less than 4 mg/L of ampicillin and in-between 4 and 8 mg/L of ampicillin, respectively. As shown in Figure 4.4, MIC_{50} and MIC_{90} for 10 mg Cl_2/L were less than or equal to 4 mg/L and in-between 4 and 8 mg/L of ampicillin, respectively.

From the observations of these graphs, it can be inferred that *E. coli* was more resistant to low concentration of chlorine, which was 1 mg/L in this study, because a higher ampicillin concentration, which was greater than 16 mg/L in this study, was required in order to decrease the number of *E. coli* colonies even by 50%.

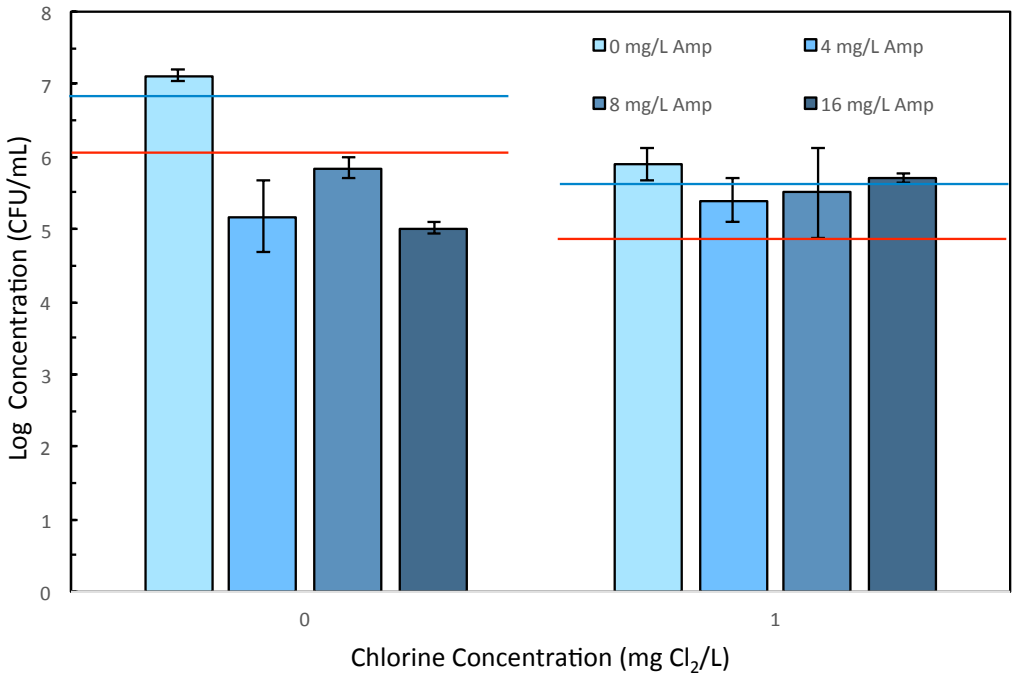

Figure 4.2. Average concentrations of control and ampicillin-resistant bacteria after treatment with a 1 mg/L of chlorine concentration with a 24-h standing period (reactivation). The blue line and the red line indicate 50% and 90% reduction of bacterial concentration at 0 mg/L of ampicillin, respectively.

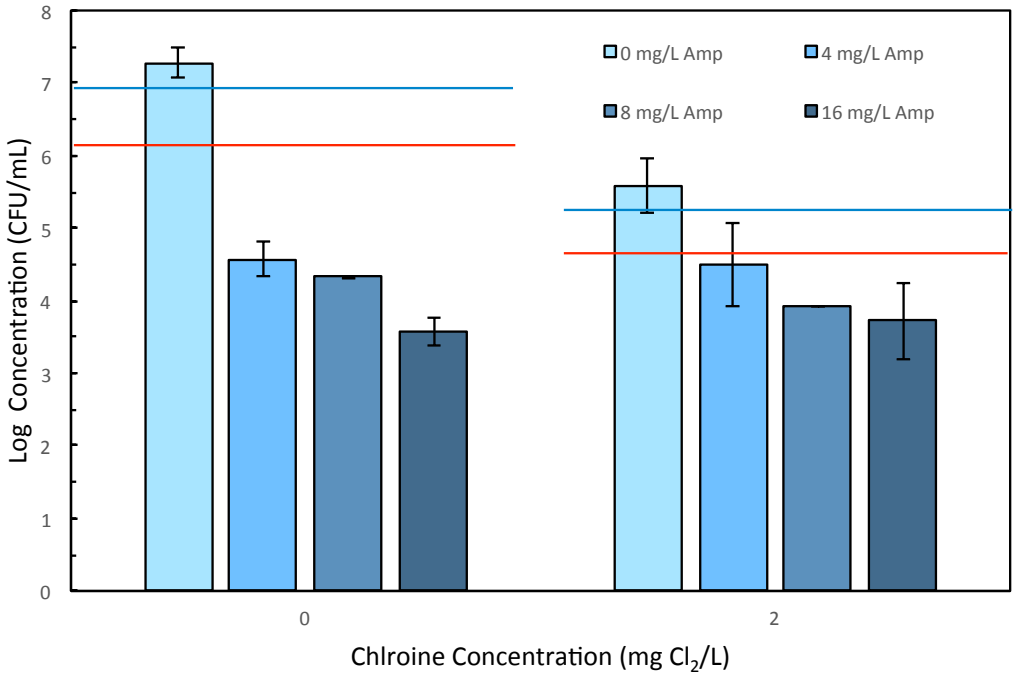

Figure 4.3. Average concentrations of control and ampicillin-resistant bacteria after treatment with a 2 mg/L of chlorine concentration with a 24-h standing period (reactivation). The blue line and the red line indicate 50% and 90% reduction of bacterial concentration at 0 mg/L of ampicillin, respectively.

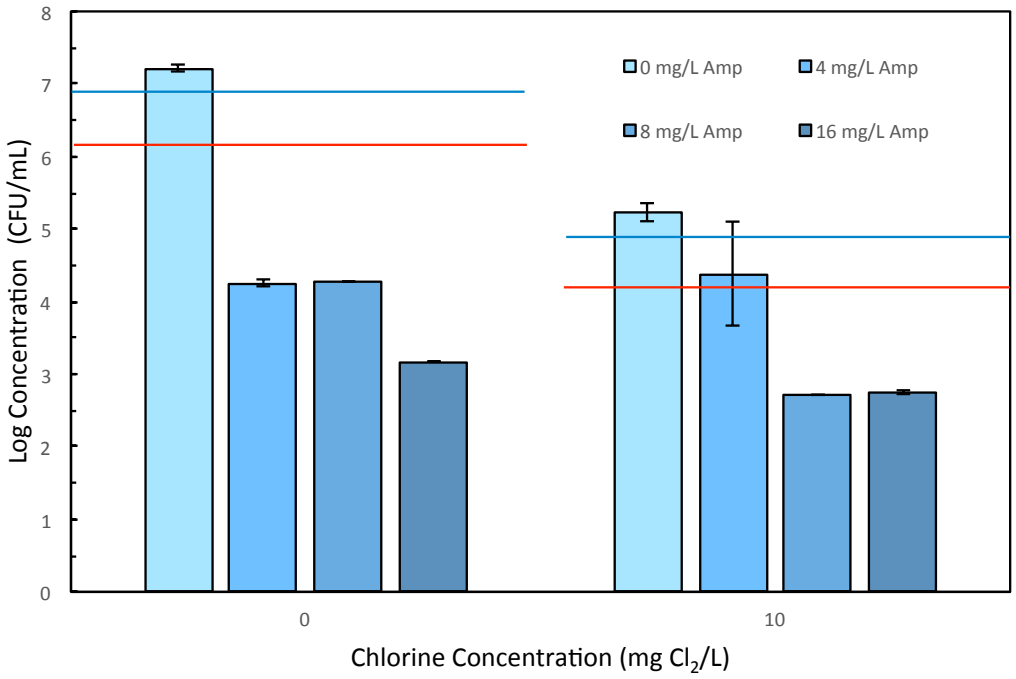

Figure 4.4. Average concentrations of control and ampicillin-resistant bacteria after treatment with a 10 mg/L of chlorine concentration with a 24-h standing period (reactivation). The blue line and the red line indicate 50% and 90% reduction of bacterial concentration at 0 mg/L of ampicillin, respectively.

	0 mg Cl_2/L*	1 mg Cl_2/L	2 mg Cl_2/L	10 mg Cl_2/L
MIC_{50} (mg/L ampicillin)	0-4	16+	0-4	0-4
MIC_{90} (mg/L ampicillin)	0-4	16+	4-8	4-8

Table 4.1. Minimum inhibitory concentration of ampicillin at different chlorine concentrations. *MIC_{50} and MIC_{90} results are the same for all 0 mg Cl_2/L experiment

Average percentages of ampicillin-resistant bacteria concentrations relative to bacteria concentrations for 0 mg/L ampicillin before and after chlorination with a 24-h retention time are shown in Table 4.2, Table 4.3, and Table 4.4. These values demonstrate the change in *E. coli* concentrations before and after chlorination. Average percentages of viable ampicillin-resistant bacteria concentrations after 1 mg Cl_2/L for ampicillin concentrations of 4, 8, and 16 mg/L were 45.06%, 61.38%, and 81.35%, respectively, while those after 0 mg Cl_2/L for ampicillin concentrations of 4, 8 and 16 mg/L were 1.08%, 5.33%, and 0.83% (Table 4.2). Average percentages of viable ampicillin-resistant bacteria concentrations after 2 mg Cl_2/L for ampicillin concentrations of 4, 8, and 16 mg/L were 8.99%, 3.73%, and 1.55%, respectively, while those after 0 mg Cl_2/L for ampicillin concentrations of 4, 8 and 16 mg/L were 0.26%, 0.13%, and 0.02% (Table 4.3). Average percentages of viable ampicillin-resistant bacteria concentrations after 10 mg Cl_2/L for ampicillin concentrations of 4, 8, and 16 mg/L were 17.20%, 0.26%, and 0.35%, respectively, while those after 0 mg Cl_2/L for ampicillin concentrations of 4, 8 and 16 mg/L were 0.11%, 0.11%, and 0.01% (Table 4.4). These results indicated that resistance of *E. coli* to ampicillin was strengthened after chlorination and *E. coli* were the most resistant when they were chlorinated at the lowest chlorine concentration.

		Ampicillin Concentration (mg/L)		
		4	8	16
Chlorine Concentration (mg Cl_2/L)	0	1.08 %	5.33 %	0.83 %
	1	45.06 %	61.38 %	81.35 %

Table 4.2. Average percentage of viable ampicillin-resistant bacteria concentrations relative to bacterial concentrations at 0 mg/L of ampicillin after 1 mg/L of chlorination with a 24-h dark period.

		Ampicillin Concentration (mg/L)		
		4	8	16
Chlorine Concentration (mg Cl_2/L)	0	0.26 %	0.13 %	0.02 %
	2	8.99 %	3.73%	1.55 %

Table 4.3. Average percentage of viable ampicillin-resistant bacteria concentrations relative to bacterial concentrations at 0 mg/L of ampicillin after 2 mg/L of chlorination with a 24-h dark period.

		Ampicillin Concentration (mg/L)		
		4	8	16
Chlorine Concentration (mg Cl_2/L)	0	0.11 %	0.11 %	0.01 %
	10	17.20 %	0.26 %	0.35 %

Table 4.4. Average percentage of viable ampicillin-resistant bacteria concentrations relative to bacterial concentrations at 0 mg/L of ampicillin after 10 mg/L of chlorination with a 24-h dark period.

4.2 Discussion

Figure 4.1 represented the inactivation of *E. coli* by chlorination. From the graph, it can be inferred that chlorination is effective in inactivating *E. coli*, and ampicillin, in addition to chlorine, can inactivate *E. coli* almost completely. After leaving the samples in the dark for 24-h for reactivation, however, average ratios of ampicillin-resistant bacteria concentrations were significantly greater than those for inactivation. Especially at chlorine concentration of 1 mg/L, the highest resistance was observed at each ampicillin dose (Table 4.2).

Reactivation results demonstrated that standing period after chlorination allowed ampicillin-resistant *E. coli* to survive and reproduce further. Likewise, the presence of chlorine, especially at low chlorine concentration, increased the selection of ampicillin-resistant bacteria significantly as reported by Huang et al. (2011) and Li et al. (2013). The selection of ampicillin-resistant bacteria might have been induced by several stress factors that were present in the study.

In order to prevent toxic derivatives of HOCl, phosphate buffer saline (PBS) was used in the study (Dukan and Touati, 1996). Since the samples were in PBS, they did not have sufficient nutrients to grow further. As Gottesman (1984), Jenkins et al. (1988), and Almiron et al. (1992) demonstrated, *E. coli* are one of the microorganisms that might have resisted the stress by generating a specific protein such as Dps in order to protect their DNA from starvation. In addition to this starvation as a stress, chlorine was another environmental stress to *E. coli* in this study. The combination of these two stresses might have caused the induction of resistance mechanisms of *E. coli* to a greater degree as reported by LeChevallier et al. (1988) and Jenkins et al. (1988). When *E. coli* colonies were exposed to ampicillin readily after chlorination for inactivation, it might not have been enough time for *E. coli* to fully develop their resistance to

chlorination. Therefore, ampicillin might have been capable of inactivating *E. coli* almost completely and possibly stopping the trigger of resistance. Since *E. coli* colonies counts were nearly 0 when bacteria were exposed to ampicillin immediately after chlorination, it can be inferred that the average percentage of ampicillin-resistant bacteria concentrations relative to bacterial concentrations at 0 mg/L of ampicillin was 0%. After leaving the chlorinated samples in the dark for 24 h, however, average fractions of viable ampicillin-resistant bacteria concentrations relative to bacterial concentrations at 0 mg/L of ampicillin were significantly greater than those for inactivation results. Therefore, the standing period increased the risk of reactivation of ampicillin-resistant bacteria.

4.3 Limitations of Study

There were several limitations for the study. First, the time available was not sufficient for in-depth study. Further experiments with actual wastewater samples and/or different doses of chlorine and antibiotics were not performed since it was out of the scope of this research. Further study with actual water and/or wastewater samples with varying levels of concentrations of chlorine and antibiotics should be completed to investigate the variation of results. Second, the study was completed only with pure culture of non-pathogenic *E. coli* K12 strains. Cherchi and Gu (2011) performed chlorine disinfection experiment with *E. coli* K12 and O157:H7, which is known as an enterhemorrhagic strain, in order to observe the inactivation behavior of *E. coli* strains by chlorination. The results demonstrated that *E. coli* O157:H7 strain is more susceptible to chlorine than *E. coli* K12 strain for all growth phases. Therefore, the pathogenic strain may be more sensitive to chlorine than the lab strain. Since different bacterial strains may have varying susceptibility to chlorination, more studies using different bacteria strains should be investigated

in order to protect the public health. Third, the study was completed with one antibiotic, which was ampicillin. Therefore, the study may not be relevant to every antibiotic and different antibiotics that are common in water and wastewater stream should be investigated. Lastly, the chlorinated samples were neutralized with sodium thiosulfate in this study so there was no residual chlorine as in actual water storage or distribution system. The results from this study, therefore, may not represent the actual condition. Further studies should be completed with chlorine residual to see the behavior of antibiotic-resistant bacteria.

CHAPTER V: CONCLUSIONS AND RECOMMENDATIONS

The inactivation results demonstrated that chlorination reduces the concentration of *E. coli* K12, and the combination of chlorine and ampicillin can inhibit *E. coli* concentrations by nearly 100%. However, when the chlorinated samples were left in the dark for 24 hours, the detection of ampicillin-resistant *E. coli* was observed. The fraction of viable cells for reactivation was significantly higher after treatment with low chlorine concentration, which was 1 mg Cl_2/L in this study.

The EPA states that chlorine concentration for typical wastewater treatment ranges from 5 to 20 mg Cl_2/L (USEPA, 1999; Lazarova et al. 1999). The study of this thesis demonstrated that ampicillin-resistant bacteria reactivated at low chlorine concentration, especially at 1 mg Cl_2/L, thus it indicated that low chlorine doses might not be sufficient to prevent the reactivation of ampicillin-resistant bacteria in a longer run. The ranges of chlorine dose for typical treatment facilities are well above the low doses of chlorine such as 1 and 2 mg Cl_2/L tested in the study of this thesis, and hence viable ampicillin-resistant bacteria concentrations in the actual system might not be as high as the ones after 1 mg Cl_2/L in the study. However, MIC_{90} for 10 mg Cl_2/L was in-between 4 and 8 mg/L to inhibit the bacteria by 90% and the average percentage of viable ampicillin-resistant bacteria concentration after 10 mg Cl_2/L for ampicillin concentration of 4 mg/L was 17.20%. This indicated that high ampicillin concentration is required in order to inhibit the reactivation of ampicillin-resistant bacteria that survived during retention time after chlorination with high chlorine concentration. Likewise, the risk of ampicillin-resistant bacteria regrowth can be still expected for the chlorination doses of typical wastewater disinfection.

Therefore, further studies on the level of antibiotics in aqueous environment and effects of chlorination on ARB should be investigated.

The EPA has set a MRDL of chlorine as 4 mg/L and the minimum chlorine residual concentration as 0.2 mg/L at point-of-entry (POE). Water Services Authorities and private water suppliers ensures that at least 0.1 mg/L free residual chlorine is maintained at the extremities of the distribution system where residual chlorine levels are likely to be at their lowest (USEPA, 2013). Since the chlorine was neutralized with sodium thiosulfate in the study of this thesis, these residual chlorine concentrations have not been verified to be safe limit for ARB. Therefore, further studies by emulating the similar conditions of discharge or distribution system from water and wastewater treatment facilities are needed to ensure the environment and public safety.

This study will be crucial for public health safety concerns because disinfection is required to control the waterborne pathogens for reclaimed and reused water, and chlorination is still the most widely used disinfection method for water and wastewater. In order to protect the public health, further evaluation of the efficiency of chlorination on ARB is needed.

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