Title

Effects of chlorination on the inactivation and reactivation of Escherichia coli K12 and its ampicillin resistance gene

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ABSTRACT OF THESIS

Effects of chlorination on the inactivation and reactivation
of *Escherichia coli* K12 and its ampicillin resistance gene

by

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

Antibiotic resistance genes (ARGs) and antibiotic resistant bacteria (ARB) are an emerging threat to public health and are identified as contaminants in aquatic environments. This study investigated the effect of chlorine on the inactivation and reactivation of *Escherichia coli* K12 and its ampicillin resistance gene. Inactivation of K12 was achieved with exposure to chlorine for 10 minutes, which were then allowed to reactivate in the dark for 24 hours. Bacterial populations were enumerated using heterotrophic plate counts containing concentrations of ampicillin, which caused further inactivation. The results showed that nearly 100% of the bacterial population was inactivated by ampicillin regardless of chlorination. Upon reactivation, a sharp decrease in bacterial concentration is observed between the control (0 mg/L Ampicillin) and any ampicillin concentration in unchlorinated samples. However, after chlorination, there is a much less decrease, suggesting that bacteria that survived chlorination were more resistant to

ampicillin. Both inhibitory concentrations (MIC_{50} and MIC_{90}) were greater than 16 mg/L Amp for at 1 mg/L Cl_2. At higher chlorine concentrations, the MIC_{90} shifted from 0-4 mg/L to 4-8 mg/L ampicillin.

The thesis of Kevin Ho is approved.

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1.0 Background and Literature Review

There is growing concern worldwide about the emergence of antibiotic resistance genes (ARGs) and bacteria (ARB) in aquatic environments. With the ease and convenience of traveling across international borders, antibiotic resistance is spreading at a remarkable speed. Each year in the United States, at least 2 million people get infections from pathogenic bacteria that are resistant to one or more of the antibiotics designed to treat those infections (CDC 2013). Bacteria can often acquire resistance from the widespread use of antibiotics. The misuse and overuse of antibiotics in both human medicine and animal husbandry is one of the important reasons for the increase and spread of ARGs and ARBs in the environment (Yang et al., 2011, Zhuang et al., 2015). While disinfection from wastewater treatment plants (WWTPs) can inactivate some ARB and ARGs, there are still many that have been found in various aquatic environments. ARGs have been recognized as emerging environmental contaminants due to their increasing abundance and imminent threat (Pruden et al., 2006).

1.1 Antibiotics in the Environment

Antibiotics inactivate pathogenic bacteria through disabling membrane function, enzyme activity, or other means to halt bacteria reproduction. Normally, antibiotics are used to inactivate the pathogenic bacteria when people get infections. However, misuse and overuse of antibiotics can lead to proliferation of resistance. For example, if a user of antibiotics does not take the full dose, the antibiotics are able to kill most of the bacteria, but some resistant bacteria survive. Further, even proper use of antibiotic creates resistance through the survival of bacteria after

being exposed to an antibiotic dose. The resistant bacteria are now allowed to multiply and can pass on their resistance genes to other bacteria through horizontal gene transfer.

The environmental exposures to antibiotics contribute to the development of resistance. Antibiotics disposed into the environment eventually make their way to treatment plants that treat wastewater before its final destination to the ocean. Because treatment plants receive water from many different sources, they can be a site for many diluted antibiotics. Exposure to low concentrations of antibiotics can effectively select for resistance (Munir et al. 2011, Jury et al., 2011, Yang et al., 2012). Even though concentrations of ARGs and ARB in raw wastewater are significantly reduced with wastewater treatment, high concentrations could still be present in the treated wastewater (Munir et al., 2011).

1.2 Agricultural Uses

Agriculture is one of the major uses of antibiotics. Agricultural areas and areas impacted by humans showed higher concentrations of ARB and ARGs (Munir et al., 2011). Due to the rising demand for food at a low cost, farmers use antibiotics on livestock for growth promotion and disease prevention. However, the use of antibiotics for growth promotion is not necessary. In 1999, Denmark banned the use of antibiotics on pigs and reported that there was little impact on the nation's pork industry (Levy, 2014). The antibiotic use per kilogram of pig raised dropped more than 50% while the overall productivity increased (Levy, 2014). Additionally, there was a decline in the levels of disease in pigs since antibiotics used were no longer creating selective pressures for resistance. Without the use of antibiotics, farmers combatted disease by decreasing the intense confinement animals were subjected to.

Animals that are given antibiotics to promote growth are often underfed the doses of antibiotics necessary to kill all harmful pathogens. As a result, bacteria developing resistance to these antibiotics grow in the livestock and remain on the animals after death. If not handled properly, bacteria can spread to humans. Antibiotic resistant bacteria can also spread through fertilizer or water containing animal feces. Waters contaminated by bacteria are of great concern because there is potential for transfer of antibiotic resistance to a pathogenic species (Meckes 1981). ARBs in the animal feces can remain on vegetable crops and can be potentially transferred to humans.

1.3 Spread into the Environment

Large amounts of antibiotics are released into wastewater due to disposal of unused antibiotics. ARGs can be partially removed through traditional wastewater treatment processes, but there are still a large number that pass through the treatment plant (Zhang et al., 2015). Effluents from WWTPs are recognized as sources of ARGs and ARB release into the environment (Zhuang et al., 2015). Figure 1 shows the different environmental compartments by which ARGs and ARB can be transmitted (Huijbers et al., 2015).

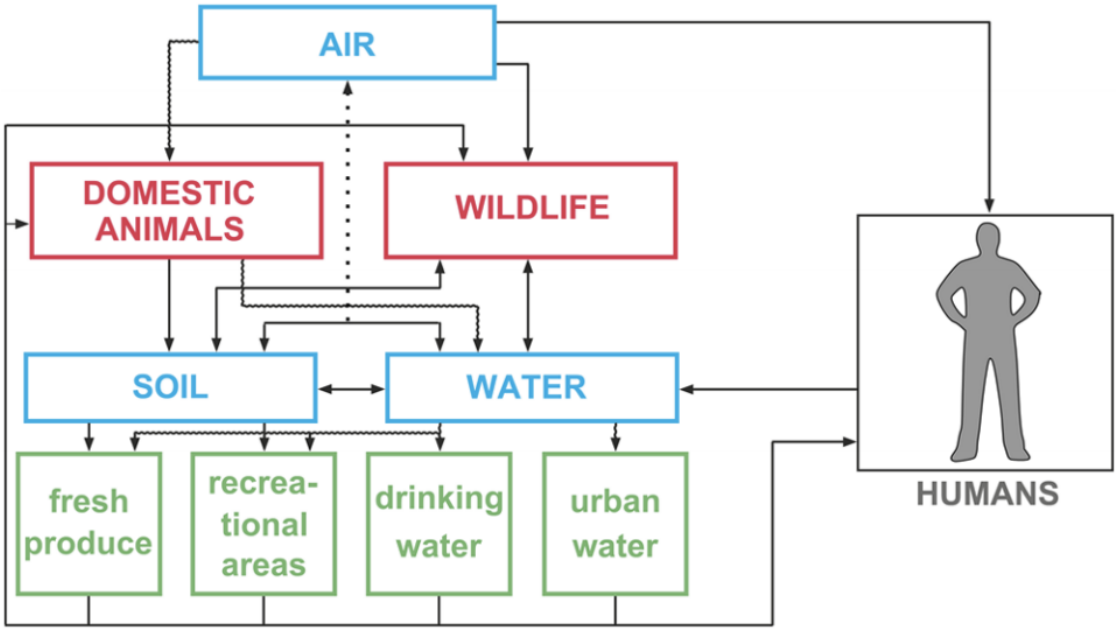

Figure 1 Transmission mechanisms of ARGs into the environment (Huijbers et al., 2015)

ARGs detected in effluents from WWTPs are at levels far above those in the typical aquatic environment (McKinney and Pruden, 2012). It is important to limit the ARG and ARB in effluents from WWTPs to reduce the spread of ARGs into the environment. ARG and ARB have been detected in wastewater samples (Zhang et al., 2009, Auerbach et al., 2007, Pruden et al., 2006). For example, beta-lactam resistance is detected in surface water (Henriques et al., 2006), drinking water, and wastewater (Schwartz et al., 2003). Furthermore, many research papers have found antibiotic resistance when dealing with wastewater.

1.4 Chlorine Disinfection

Chlorination is one of the most common disinfection processes currently used in water treatment because of its simple application and moderate cost. Free chlorine works as a nonspecific oxidant that affects a range of subcellular compounds and metabolic processes. It

affects membrane permeability, inhibits transport, fragments proteins, and reacts with nucleotides (Albrich and Hurst, 1982). In addition, chlorine inactivates enzymes, inhibits ATP production by the oxidation of ATP-synthase, and denatures nucleic acids.

Disinfection is often used to inactivate most pathogens in WWTP effluents before they are discharged into receiving waters. This disinfection process generally effectively decreases the concentration of ARBs. However, intact remnants of DNA may survive and confer ARGs to downstream bacteria by transformation and/or transduction even if ARB are fully inactivated (Dodd, 2012). Therefore, it is crucial to evaluate the effects of disinfection on the inactivation and destruction of ARGs.

Several studies reported that disinfection through chlorination and ultraviolet (UV) could not achieve significant reductions of ARGs. Munir et al. (2011) showed that chlorine and UV disinfection did not prove to have significant contribution to ARG and ARB reduction. There was little change in concentration of ARGs and ARB between pre and post disinfected effluents in multiple treatment plants (Munir et al., 2011). Although the concentrations of ARGs and ARB were reduced, there was still high concentrations in the effluent, showing that disinfection may not be sufficient in removing ARGs from wastewater. Munir et al. (2011) concluded that the disinfection process did not contribute to significant reductions, which also suggests that the discharge of final effluent can be a potential route of entry into the environment.

However, other studies observed notable inactivation of laboratory cultured ARGs or ARBs (McKinney and Pruden 2012, Huang et al., 2013). While Huang et al. (2013) noted that it is possible for antibiotic resistance to protect against chlorine and UV exposure, a 1mg/L chlorine dose was able to achieve 4-5 log inactivation. McKinney and Pruden (2012) revealed that a low UV dose of 10 mJ/cm^2 was sufficient to inactivate ARB, while a dose at least 1 order

of magnitude greater than that for inactivation of ARB was needed to inactivate ARGs. Research has shown that conventional wastewater purification methods without disinfection are not adequate for removal of ARB (Grabow et al., 1974). Wastewater disinfection is a means of limiting the number of ARB in the environment although additional studies are needed to understand the effectiveness of standard disinfection on ARGs and ARB.

The differences between the actual WWTP operation reports and laboratory research may be due to the variations of the predominant ARGs, ARB, and disinfectant dosages applied. Disinfection doses in WWTPs are not always given. It is important to compare destruction of ARGs in real wastewater effluents under specific disinfection operations to find the potential pathway to decrease the spread of ARGs into natural waters.

1.4.1 Breakpoint Reaction

During the chlorination process, ammonia nitrogen (NH_3-N) is an important parameter that affects chlorination disinfection efficiency because its rapid reaction to form combined chlorine (Zhang et al., 2015). After the addition of chlorine in WWTPs, chlorine might be quickly consumed by NH_3-N to generate monochloramine. The reactivity of combined chlorine is weaker than that of free chlorine, indicating that free chlorine is more effective than combined chlorine on the removal of ARGs.

Breakpoint chlorination describes the interaction between chlorine and ammonia-nitrogen. When chlorine is first introduced into a system, NH_3-N quickly reacts with free chlorine and forms combined chlorine such as monochloramine and dichloramine (Dodd 2012). The multiple chemical reactions between chlorine and ammonia are shown below.

$$Cl_2 + H_2O \longrightarrow HOCl + HCl \quad (1)$$

$$NH_3 + HOCl \longrightarrow NH_2Cl + H_2O + H^+ \quad (2)$$

$$NH_2Cl + HOCl \longrightarrow NHCl_2 + H_2O \quad (3)$$

$$NHCl_2 + HOCl \longrightarrow NCl_3 + H_2O \quad (4)$$

$$2\,NH_2Cl + HOCl \longrightarrow N_2 + 3\,HCl + H_2O \quad (5)$$

According to breakpoint chlorination chemistry, monochloramine is predominately formed when Cl_2:NH_3-N ratio is less than 5:1 (Equation 2). As the ratio increases above 5:1, the excess free chlorine reacts with the chloramine to form dichloramine and trichloramine (Equations 3 and 4), or reacts completely to convert ammonia to nitrogen gas (Equation 5). The latter is referred to as the "breakpoint reaction". Theoretically, once the mass ratio of chlorine to NH_3-N exceeds 7.6:1, all the ammonia-nitrogen would have been converted to nitrogen gas. At this point, any additional dose of chlorine remains as available chlorine in the water. Free chlorine dosage over the breakpoint dose (the mass ratio of chlorine to NH_3-N is 7.6:1) is required to show higher removal efficiency considering the presence of ammonia in wastewater (Zhang et al. 2015). Other factors that can affect chlorine disinfection efficiency is pH. Li and Zhang (2012) revealed that pH significantly affects the removal efficiency of chlorine disinfection.

It has been generally accepted that the amount of chlorination needed to create inactive ARGs in wastewater varies with the quality of the effluent (Zhuang et al., 2015). Different amounts of chemical oxygen demand (COD), NH_3-N, and organic matter can make wastewater more difficult to inactivate. During chlorination, the quantity of NH_3-N present in the wastewater plays a critical role in the removal of ARGs, higher NH_3-N concentration leads to lower ARG

removal, which may attribute to its rapid competition for free chlorine to form combined chlorine, such as monochloramine.

1.5 Bacterial Ability to Survive

It has been observed that there is sometimes a greater proportion of ARB in treated water and wastewater effluent compared to the proportion in untreated water, suggesting that treatment may somehow select for antibiotic resistance (Armstrong et al., 1982). Several explanations proposed some special tolerance to chlorine or protective capsule formation by antibiotic resistant bacteria (Templeton et al., 2009). One example is bacterial ability to enter a viable but nonculturable (VBNC) state under treatment processes and reactivate when environments are less stressful.

Bacterial resistance to chlorine has been observed in both lab studies and full-scale water and wastewater treatment (Cherchi and Gu, 2011). Bacteria exposed to stress environments such as starvation or heat shock can stimulate the synthesis of unique proteins to develop resistance to various disinfection methods. It is suggested that the different operating conditions of WWTPs can dictate the chlorine inactivation kinetics of bacterial populations (Cherchi and Gu 2011, Li et al., 2013). Li et al. (2013) noted that bacteria cultivated under low nutrient conditions exhibited diverse physiology and different susceptibilities toward disinfectants compared to cells cultivated in rich mediums. Cherchi and Gu (2011) observed *E. coli* that were exposed to chlorine produced a specific set of proteins that made them less susceptible to the disinfectant.

1.6 Ampicillin

Ampicillin is a commonly used antibiotic used to combat infections. It belongs to the penicillin group of bactericidal beta-lactam antibiotics, which are effective against Gram-positive bacteria and some Gram-negative bacteria such as *E. coli*. The beta-lactam antibiotics generally induces cell lysis by competitive inhibition of the transpeptidase enzymes that are used during cell wall synthesis (Templeton et al., 2009). Ampicillin inhibits the formation of peptidoglycan cross-linkages in the cell wall (Huang et al., 2011).

The main modes of resistance to ampicillin is the hydrolysis of the antibiotic beta-lactam ring by the bacterial enzyme beta-lactamase (CDC, 2013). This disables ampicillin from being able to bind to penicillin-binding enzymes (Templeton et al., 2009). Also, the beta-lactamase can destroy ampicillin before the antibiotic can take effect. Beta-lactamase is often found in bacterial chromosomes and may be transferred as plasmids.

1.6.1 Minimum Inhibitory Concentration

The minimum inhibitory concentration (MIC) is the lowest concentration of an antibiotic to inactivate a certain percentage of the bacterial population. MIC_{50} and MIC_{90} are often used to describe the concentration needed to inactivate 50% and 90% of the population, respectively. The MIC_{50} is a conventional indicator to determine the antibiotic resistance of bacteria in the field of medical research (Huang et al., 2013).

1.7 Gene Transfer

In addition to mutation, there are two routes for acquiring resistance, vertical inheritance or horizontal transfer. Vertical inheritance is determined by natural selection where a random

mutation can result in resistance that is then passed down through its progeny. As shown in Figure 2, horizontal transfer can occur through conjugation (direct contact transfer of plasmids), transduction (injection of DNA into bacteria through bacteriophages), or transformation (DNA uptake) (Dodd 2012, Jury et al., 2011). In water treatment processes, ARGs are usually transferred through transduction and natural transformation (Pang et al., 2015).

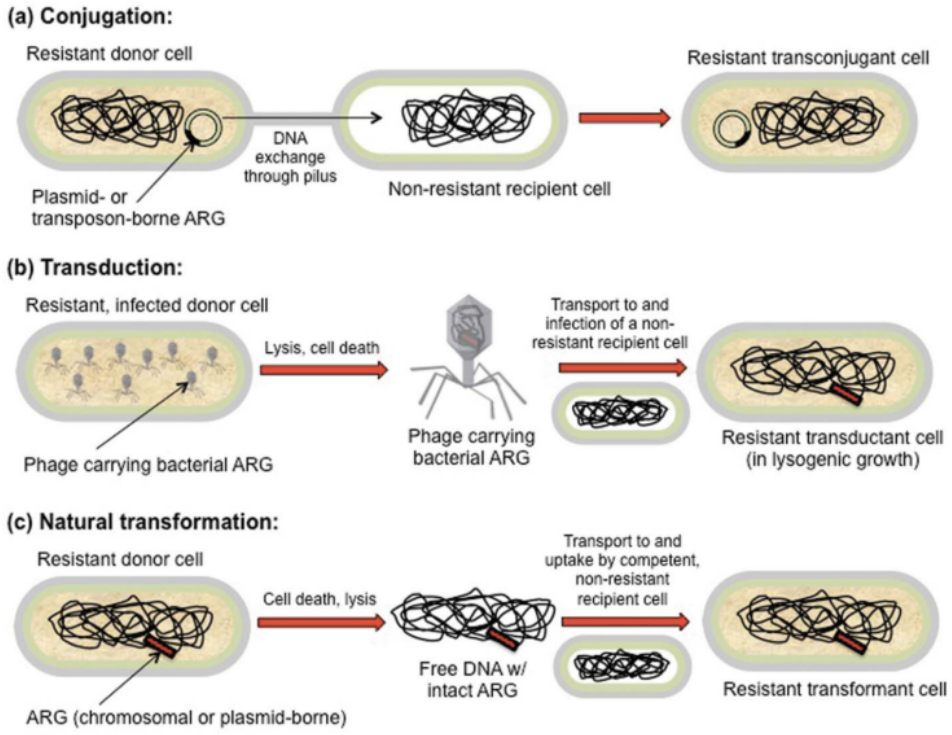

Figure 2 Horizontal gene transfer mechanisms: a) conjugation, b) transduction, and c) natural transformation (Dodd 2012)

In order for resistance genes to be transferred, they must be in chromosomes, R plasmids, or integrons (Jury et al., 2011). Bacteria carrying transmissible R-factors are responsible for the spread of multiple antibiotic resistance (Meckes 1981). The transmission of R-factors in *Enterobacteriaceae* usually occurs by conjugation, which involves a specialized structure called

the “sex pilus” and requires cell to cell contact (Meckes 1981). Transmission of R-factors by conjugation is rapid and may rapidly spread resistance genes to other bacteria.

When bacteria carrying transmissible R-factors are ingested by a host, the R-factors may be transferred into the bacteria commonly found in the gastrointestinal tract (Meckes 1981). These organisms can further transfer the resistance to pathogenic organisms, resulting in reduced efficiency of antibiotics. A single genetic cassette can often contain many different resistance mechanisms (Jury et al., 2011). As a result, it is possible to become multi-resistant by a single genetic uptake.

The abundance of genes in aquatic environments have the potential to result in bacteria that are resistant to many types of antibiotics. Watanabe (1962) discovered in that *Escherichia coli* strains could transfer antibiotic resistance to antibiotic sensitive strains. Further research demonstrated that the transfer of antibiotic resistance genes has created superbugs such as *Klebsiella pneumonia* harboring NDM-1 gene (Yong et al., 2009).

Persister cells are surviving cells that neither grow nor die in the presence of a disinfectant. They differ from resistant cells in that persister cells are unable to grow while exposed to a disinfectant while a resistant cell can. However, persister cells can survive disinfection and potentially absorb external genetic material and develop resistance. While the mechanisms involved are not fully understood, they may be responsible for high levels of resistance (Cherchi and Gu, 2011).

1.8 Regrowth/Reactivation

Even after chlorination, there are still microbial risks associated with bacterial exposure. Research indicates that bacteria are able to reproduce in distribution systems after chlorination

(Huang et al., 2011). There are limited studies that investigate the reactivation of antibiotic resistant bacteria after chlorination. Murray et al. (1984) looked at the proportions of 11 different kinds of antibiotic resistant bacteria between chlorinated samples and chlorinated samples neutralized by sodium thiosulfate after 24 hours. They found that there was an increased proportion of antibiotic resistant bacteria after the standing period of 22-24 hours after termination of chlorination.

The regrowth of bacteria includes the regrowth of living bacteria, reactivation of inactivated bacteria, and the regrowth of reactivated ones (Huang et al., 2011). Reactivation of bacteria was most likely to occur after exposure to low doses of chlorination (Li et al., 2013). Although chlorine disinfection can reduce regrowth and reactivation, bacteria still have the potential to regrow.

2.0 Investigation of Bacterial Disinfection and Regrowth with a Pure Culture

In this study, a pure *Escherichia coli* (*E. coli*) K12 was chosen to analyze the effects of chlorination on ARB. *E. coli* K12 was used because a pure culture of bacteria was needed in order to minimize variability. We wished to determine how chlorination affected the minimum inhibitory concentration (MIC) of the *E. coli* K12 population. First, the *E. coli* strain was exposed to different chlorine concentrations. Then, the *E. coli* populations were exposed to different ampicillin concentrations in nutrient agar to determine shifts in ampicillin resistance. The *E. coli* populations were also left in the dark to simulate water in a pipes carried to a

destination or in retention basins. *E. coli* is suspected to potentially reactivate during this time and can cause further shifts in MIC.

2.1 Methods

E. coli K12 was grown in nutrient broth at 25°C. The bacteria were transferred to new nutrient broth media (3.0 g/L beef extract, 5.0 g/L peptone) approximately every two to three days to keep the *E. coli* in exponential phase. Each transfer was diluted by serial 10-fold dilutions. On days of experimentation, the bacteria were transferred to new media the day before, regardless of when the bacteria were previously transferred. This is to ensure that the K12 population is in exponential phase instead of potentially being in stationary phase.

The cells were collected by centrifugation (7280 rpm, 15 min, 4°C). The settings were based on Pang et al. (2015) study of 10000 rpm for 10 min at 4°C. The bacteria were washed three times with sterile phosphate-buffered saline (PBS, pH=7.4) water, and subsequently suspended in PBS. The washing step is required to remove the nutrient broth that the bacteria were growing in. This prevents the *E. coli* from further growing throughout the experiment. Washing also prevents nutrient broth from interfering with chlorine disinfection. The ammonia in the nutrient broth can react with the free chlorine to create monochloramine. Following the washing step, the ammonia concentration in NH3-N was measured using Hach Ammonia TNT method (Loveland, CO). The final concentration of suspended *E. coli* K12 cultures were approximately 10^7 colony forming units (CFU)/mL.

2.2 Chlorination

PBS samples of *E. coli* K12 were transferred into sterile 250 mL flasks and were mixed with a magnetic stir bar to mix the sample at room temperature (25°C). Sodium hypochlorite was added at a range of available chlorine doses of 0, 1.0, or 10.0 mg/L, and were in contact with the bacteria for 10 minutes. Chlorine was measured after 10 minutes to determine the chlorine demand of the bacteria and solutoin. A sodium thiosulfate solution (1.5%) was added in excess to terminate the chlorination process and destroy the remaining chlorine residual. Volume was extracted in order to perform plate counts. After the addition of sodium thiosulfate, the flasks were covered in foil and placed in a dark environment for 24 hours at room temperature. Each chlorination disinfection of *E. coli* K12 was performed in duplicate.

2.3 Plating

The ampicillin resistance of *E. coli* K12 was determined by exposing the samples to serial ampicillin concentrations in nutrient agar. Nutrient agar plates were made with different ampicillin concentrations (0, 4, 8, 16 mg/L). 50 µL of each sample was placed on 90mm Petri dishes containing the nutrient agar with different ampicillin concentrations. To obtain colony counts between 30 and 300 per plate, all samples were diluted by serial 10-fold dilutions in PBS. The plates were then incubated at 37°C for 24 hr. Plating was conducted two times: before and after the 24 hour dark period.

2.4 Results

The inactivation of *E. coli* K12 by chlorination is shown in Figure 3 to determine the bacterial response to chlorination. A separate experiment was done to determine the inactivation profile of *E. coli* K12.

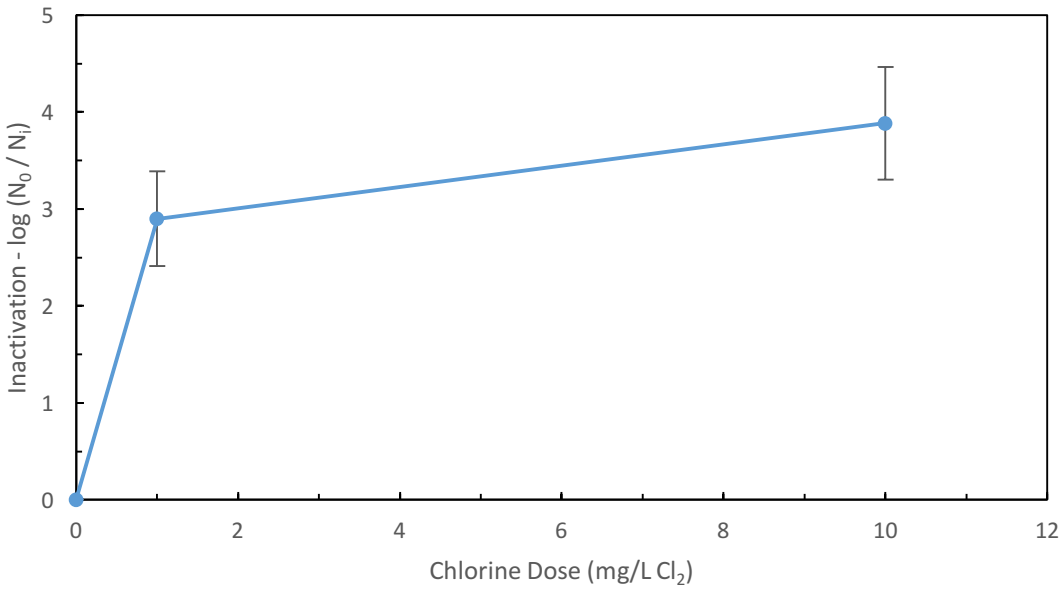

Figure 3 Inactivation of *E. coli* K12 by chlorination. The results show the average of one chlorination experiment with four replicates. Error bars indicate standard deviation for replicates from a single sample

The inactivation shows the high effectiveness of chlorine on the *E. coli* population. A dose of 1 mg/L was sufficient for a 3-log inactivation. As the chlorine concentration increased to 10 mg/L, there was a 1-log increase relative to the 1 mg/L chlorine dose. This inactivation curve was similar to the curve reported by Pang et al. (2015) and Huang et al. (2011, 2013), which showed the inactivation curve level off around 3-4-log.

When testing inactivation by antibiotic, we found that the lowest dose of ampicillin was enough to nearly inactivate the entire K12 population, regardless of chlorination. At 4 mg/L Amp, the colonies counts were far below the preferred range of 30-300 colonies even in the undiluted samples. Therefore, we suggest that an ampicillin dose of 4 mg/L was effective to inactivate the *E. coli* population.

Following reactivation, it was observed that the *E. coli* population reactivated after being left in the dark for 24 hours. The reproduction of bacteria after chlorination includes the reactivation of inactivated bacteria and regrowth of reactivated ones. Below are the results for three separate experiments testing different concentrations of chlorine and ampicillin. These bacterial concentrations shown are representative of samples left in the dark for 24 hours after inactivation by chlorination. Samples were inactivated by chlorine, followed by ampicillin. There are no data for inactivation because any concentration of ampicillin inactivated the *E. coli* population such that bacterial concentrations were not measurable.

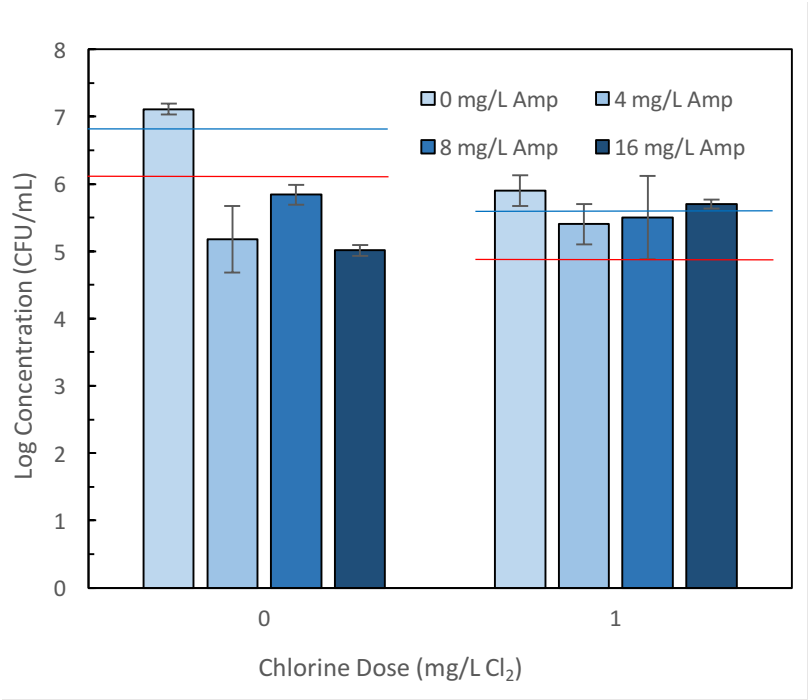

Figure 4 Concentration of *E. coli* K12 after inactivation with a 1 mg/L chlorine dose and 24 hour dark period. The initial concentrations of *E. coli* were 10^7 CFU/mL and the contact time was 10 minutes. The blue and red lines represent 50% and 90% of the 0 mg/L Amp bacterial concentrations, respectively. Error bars indicate standard deviation for replicates from a single sample

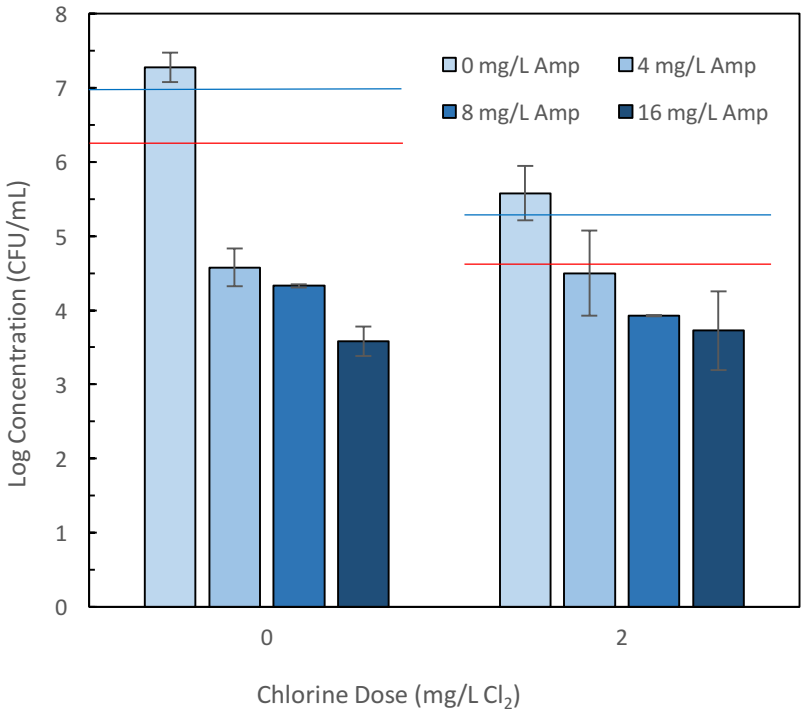

Figure 5 Concentration of *E. coli* K12 after inactivation with a 2 mg/L chlorine dose and 24 hour dark period. The initial concentrations of *E. coli* were 10^7 CFU/mL and the contact time was 10 minutes. The blue and red lines represent 50% and 90% of the 0 mg/L Amp bacterial concentrations, respectively. Error bars indicate standard deviation for replicates from a single sample

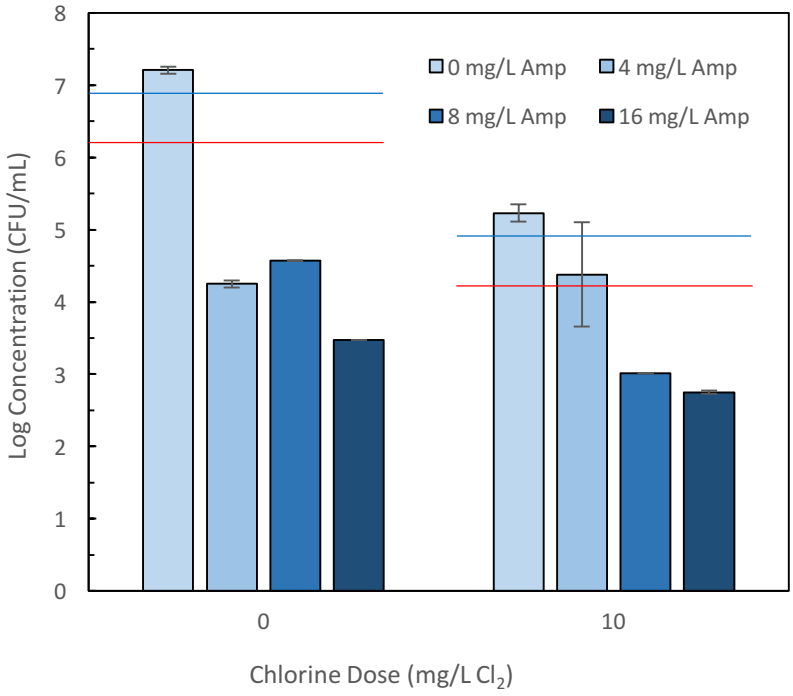

Figure 6 Concentration of *E. coli* K12 after inactivation with a 10 mg/L chlorine dose and 24 hour dark period. The initial concentrations of *E. coli* were 10^7 CFU/mL and the contact time was 10 minutes. The blue and red lines represent 50% and 90% of the 0 mg/L Amp bacterial concentrations, respectively. Error bars indicate standard deviation for replicates from a single sample

In each separate unchlorinated experiment, there is a noticeable drop in bacterial concentration between 0 mg/L and 4 mg/L Amp. Table 1 shows the log inactivation of ampicillin relative to the control of 0 mg/L Amp. In the first three columns showing the unchlorinated experiments, 4 mg/L and 8 mg/L Amp had similar inactivation values, while 16 mg/L Amp was about 0.7 to 0.9 greater. This suggests that the ampicillin dose of 4 mg/L was able to inactivate the majority of the ampicillin sensitive bacteria. The bacteria that survived the 4 mg/L Amp dose

were more resistant to ampicillin and harder to kill. Evidence of this is shown by the inactivation at concentrations of 8 mg/L and 16 mg/L Amp were only marginally better than that of 4 mg/L Amp.

Table 1 Log inactivation of ampicillin relative to control (0 mg/ L Ampicillin)

	0 mg/L Cl_2 Run 1	0 mg/L Cl_2 Run 2	0 mg/L Cl_2 Run 3	1 mg/L Cl_2	2 mg/L Cl_2	10 mg/L Cl_2
4 mg/L Amp	1.9	2.7	3.0	0.49	1.1	0.85
8 mg/L Amp	1.3	2.9	2.6	0.40	1.7	2.2
16 mg/L Amp	2.1	3.7	3.7	0.19	1.9	2.5

From looking at the results after chlorination, it is possible to see chlorine resistance of the K12 population. There was less inactivation from 0 mg/L to 4 mg/L Amp after chlorination than before chlorination. In Figure 4 and 5, the the bacterial concentrations for 4, 8, and 16 mg/L Amp were relatively the same. This suggests that the low levels of chlorine did not influence the effect of ampicillin. Table 1 shows that the inactivation at 1 mg/L Cl_2 and 2 mg/L Cl_2 across all ampicillin concentrations were about 0.40 and 1.5-log averaged, respectively. Compared to 0 mg/ L Cl_2, there was much less inactivation. It is possible that the bacteria that survived chlorination were more likely to be tolerant to ampicillin. However, when the bacteria were exposed to 10 mg/L Cl_2, there was a noticeable decrease at 8 Amp and 16 mg/L Amp. It could be that the high level of chlorine only allowed mildly ampicillin tolerant bacteria to survive. The bacteria could be able to survive a 4 mg/L ampicillin dose, but not any higher concentration.

The experiments also show shift in MIC values. The MIC is a conventional indicator to determine the antibiotic resistance of bacteria (Huang et al., 2013). In Figure 4, 5, and 6, 50% and 90% of the control concentration (0 mg/L Amp) are shown in the blue and red line,

respectively. The MIC values were calculated to find any shifts in ampicillin resistance before and after chlorination. While the exact MIC is not precisely measured, it is possible to estimate it to within a range. Furthermore, MIC_{50} and MIC_{90} were determined when the concentration consistently inhibited 50% and 90%, respectively. Standard deviations and errors were taken into account to establish a conservative estimate. Before chlorination, the MIC_{50} and MIC_{90} were consistently below 4 mg/L ampicillin. At 1 mg/L Cl_2, the conclusion was that the MIC was above 16 mg/L Amp because there was no consistent inhibition. At 2 mg/L Cl_2, the MIC_{90} is between 4 and 8 mg/L Amp. At 10 mg/L Cl_2, the MIC_{90} is between 4 mg/L and 8 mg/L ampicillin. This suggests that at low chlorine concentrations, ampicillin had little to no effect.

Table 2 Minimum inhibitory concentration of ampicillin at different chlorine concentrations

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

*MIC_{50} and MIC_{90} results are the same for all 0 mg/L Cl_2 runs

3.0 Discussion

According to a critical review from Huijbers et al. (2015), ARB were detected in all publications wastewaters investigated. Wastewater that is discharged into surface water has also been observed to have high concentrations of ARB, showing high contribution of wastewater to disseminate antibiotic resistance to other environmental compartments. Recent studies showed that WWTPs can be reservoirs for ARB and ARGs (Pang et al., 2015, Zhuang et al., 2015). Heterotrophic bacteria exhibiting resistance to multiple antibiotics were detected in wastewater effluents (Pang et al., 2015). With increasing use of antibiotics, it is necessary to recognize the

effects they can have on bacteria. Increasing resistance and the ability for ARB to quickly multiply show a need for better monitoring efforts.

3.1 Selective Pressures

Treatment plants may serve as a suitable process for the increase and spread of ARGs and ARB because a high density of active bacteria promotes horizontal gene transfer (Chen and Zhang 2013). Therefore, WWTPs could increase the antibiotic resistance of surviving bacteria and serve as a reservoir for the spread of antibiotic resistance. Sewage treatment plants may have an important role in the dissemination of ARGs in the water environment.

While chlorine disinfection in treatment plants can differ, it is possible that the chlorine doses used can select for antibiotic resistance. The role of WWTPs in reducing the load of antibiotic resistant bacteria in raw sewage is not well known (Munir et al., 2011, Dodd 2012). The results from this study showed that ARB can reactivate and grow even if they are inactivated by chlorine or antibiotics. Chlorine disinfection can also select for resistance, making the surviving bacteria harder to inactivate. Due to the proportional change in ARB population exposed to chlorine, chlorination should be considered as a selective pressure for ARB (Huang et al. 2011). Sewage treatment plants could potentially increase the concentration of resistance genes during treatment due to selective pressures in sewage (Armstrong et al., 1982, Munir et al., 2011, Jury et al., 2011, Yang et al., 2012).

Furthermore, low concentrations of chlorine doses can cause ineffective disinfection. WWTPs often measure their chlorine treatments by CT values, defined as chlorine concentration multiplied by time. However, different chlorine doses may have varying results despite having the same CT value. Huang et al. (2011) demonstrated the different effects of a constant CT value

of 50 mg-min/L Cl_2. An exposure of 25 mg/L Cl_2 for 2 min was significantly more effective than a 2 mg/L Cl_2 dose for 25 min. Because there was not widespread knowledge on the specific CT values WWTPs use, this study used varying doses and a constant time. CT values were not constant, but the study was more controlled varying only the dose. From the results of the study, the low dose resulted in a higher concentration of surviving bacteria.

3.2 Limitations

The specific scope of this bench scale experiment can be limiting. The differences between actual WWTP operations and laboratory research may be due to the variations of ARGs, ARB and disinfection doses. Disinfection doses in WWTPs are not always given and the amount of chlorination needed to inactivate ARGs in wastewater varies on the quality of the effluent (Zhuang et al. 2015). This experiment was performed in a controlled environment to test the specific effects of chlorine and ampicillin. It is important to compare the inactivation of ARGs in real wastewater effluents under specific disinfection operations to find the potential pathway to decrease the spread of ARGs into natural water.

When testing the inactivation of chlorine and ampicillin on *E. coli*, it is possible that there is a co-selection mechanism, which is typically associated with a relationship between heavy metals and antibiotics. This phenomenon is the selection of multiple resistance genes when one of the genes is selected. When the *E. coli* population is exposed to chlorine, it can potentially encode for ampicillin resistance.

Furthermore, this study consisted of one species of *E. coli* and tested one antibiotic. This research could be extended by testing more species of bacteria that are common in wastewater effluent. Additional antibiotics that are common can also be tested. Tetracycline and sulfonamide

are commonly used antibiotics (Munir et al., 2011). To test antibiotics and the inactivation of genes, quantitative polymerase chain reaction (qPCR) can be applied to quantify specific antibiotic genes. Quantification can assist in determining the ARGs inactivated during chlorine disinfection.

Conclusion

ARGs and ARB are a major threat to the environment. There have been studies investigating the effectiveness of disinfectants, but few on reactivation of ARB downstream of treatment. There is a risk of selecting for resistant *E. coli* when chlorinating water. Selection of ARB by chlorination can depend on many factors, including but not limited to: antibiotic used, type of antibiotic resistance, chlorine dose, and recovery time.

The purpose of this study was to test the effectiveness of chlorine and ampicillin on inactivation, and to test any possible reactivation downstream of treatment. The results showed that there is high inactivation with both chlorine and ampicillin. However, after a dark period of 24 hours, the bacteria reactivated, showing that ARGs and ARB can potentially regrow after treatment. Chlorine was found to be selecting for ampicillin resistant bacteria. The MIC greatly shifted in low chlorine concentrations and still slightly shifted in higher chlorine concentrations. These results highlight the challenge in eliminating ARGs from waters. Therefore, further research should be done to study reactivation downstream of disinfection.

References

Albrich, J. M., & Hurst, J. K. (1982). Oxidative inactivation of Escherichia coli by hypochlorous acid. *FEBS Letters*, *144*(1), 157–161.

Armstrong, J. L., Calomiris, J. J., & Seidler, R. J. (1982). Selection of antibiotic-resistant standard plate count bacteria during water treatment. *Applied and Environmental Microbiology*, *44*(2), 308–316.

Auerbach, E. A., Seyfried, E. E., & McMahon, K. D. (2007). Tetracycline resistance genes in activated sludge wastewater treatment plants. *Water Research*, *41*(5), 1143–1151.

CDC. (2013). Antibiotic resistance threats in the United States, 2013. *Current*, 114.

Chen, H., & Zhang, M. (2013). Occurrence and removal of antibiotic resistance genes in municipal wastewater and rural domestic sewage treatment systems in eastern China. *Environment International*, *55*, 9–14.

Cherchi, C., & Gu, A. Z. (2011). Effect of bacterial growth stage on resistance to chlorine disinfection. *Water Science and Technology*, *64*(1), 7–13.

Dodd, M. C. (2012). Potential impacts of disinfection processes on elimination and deactivation of antibiotic resistance genes during water and wastewater treatment. *Journal of Environmental Monitoring*, *14*(7), 1754–1771.

Grabow, W. O. K., Prozesky, O. W., & Smith, L. S. (1974). Drug Resistant Coliforms Call for Review of Water Quality Standards. *Water Research*, *8*, 1–9.

Henriques, I., Moura, A., Alves, A., Saavedra, M. J., & Correia, A. (2006). Analysing diversity among b-lactamase encoding genes in aquatic environments. *FEMS Microbiology Ecology*, *56*(3), 418–429.

Huang, J., Hu, H., Tang, F., Li, Y., Lu, S., & Lu, Y. (2011). Inactivation and reactivation of antibiotic-resistant bacteria by chlorination in secondary effluents of a municipal wastewater treatment plant, *Water Research, 45*, 2775–2781.

Huang, J., Hu, H., Wu, Y.-H., Wei, B., & Lu, Y. (2013). Effect of chlorination and ultraviolet disinfection on tetA-mediated tetracycline resistance of Escherichia coli. *Chemosphere*, *90*, 2247–2253.

Huijbers, P. M. C., Blaak, H., Jong, M. C. M. De, Graat, E. A. M., Vandenbroucke-grauls, C. M. J. E., Maria, A., & Husman, D. R. (2015). Role of the Environment in the Transmission of Antimicrobial Resistance to Humans: A Review. *Environmental Science and Technology*, *49*, 11993–12004.

Jury, K. L., Khan, S. J., Vancov, T., Stuetz, R. M., & Ashbolt, N. J. (2011). Are Sewage Treatment Plants Promoting Antibiotic Resistance? *Critical Reviews in Environmental Science and Technology*, *41*(3), 243–270.

Levy, S. (2014). Reduced antibiotic use in livestock: How Denmark tackled resistance. *Environmental Health Perspectives*, *122*(6), 160–165.

Li, B., & Zhang, T. (2012). PH significantly affects removal of trace antibiotics in chlorination of municipal wastewater. *Water Research*, *46*(11), 3703–3713.

Li, D., Zeng, S., Gu, A. Z., He, M., & Shi, H. (2013). Inactivation, reactivation and regrowth of indigenous bacteria in reclaimed water after chlorine disinfection of a municipal wastewater treatment plant. *Journal of Environmental Sciences*, *25*(7), 1319–1325.

Mckinney, C. W., & Pruden, A. (2012). Ultraviolet Disinfection of Antibiotic Resistant Bacteria and Their Antibiotic Resistance Genes in Water and Wastewater. *Environmental Science and Technology*, *46*, 13393–13400.

Meckes, M. C. (1981). Effect of UV light disinfection on antibiotic-resistant coliforms in wastewater effluents. *Applied and Environmental Microbiology*, *43*(2), 371–377.

Munir, M., Wong, K., & Xagoraraki, I. (2011). Release of antibiotic resistant bacteria and genes in the effluent and biosolids of five wastewater utilities in Michigan. *Water Research*, *45*(2), 681–693.

Pang, Y., Huang, J., Xi, J., Hu, H., & Zhu, Y. (2015). Effect of ultraviolet irradiation and chlorination on ampicillin-resistant Escherichia coli and its ampicillin resistance gene. *Frontiers of Environmental Science & Engineering, 10*(3)*,* 522-530.

Pruden, A., Pei, R., Storteboom, H., & Carlson, K. H. (2006). Antibiotic resistance genes as emerging contaminants: Studies in northern Colorado. *Environmental Science and Technology*, *40*(23), 7445–7450.

Schwartz, T., Kohnen, W., Jansen, B., & Obst, U. (2003). Detection of antibiotic-resistant bacteria and their resistance genes in wastewater, surface water, and drinking water biofilms. *FEMS Microbiology Ecology*, *43*, 325–335.

Templeton, M. R., Oddy, F., Leung, W., & Rogers, M. (2009). A paper submitted to the Journal of Environmental Engineering and Science. *Canadian Journal of Civil Engineering*, *36*(5), 889–894.

Watanabe, T. (1962). Infective heredity of multiple drug resistance in bacteria. *Bacteriology Reviews*, *27*, 87.

Yang, Y., Zhang, T., & Zhang, X.-X. (2011). Quantification and characterization of β-lactam resistance genes in 15 sewage treatment plants from East Asia and North America. Applied Microbiology and Biotechnology, 95 (5).

Yong, D., Toleman, M. A., Giske, C. G., Cho, H. S., Sundman, K., Lee, K., & Walsh, T. R. (2009). Characterization of a new metallo-β-lactamase gene, bla NDM-1, and a novel erythromycin esterase gene carried on a unique genetic structure in Klebsiella pneumoniae sequence type 14 from India. *Antimicrobial Agents and Chemotherapy*, *53*(12), 5046–5054.

Zhang, Y., Zhuang, Y., Geng, J., Ren, H., Zhang, Y., Ding, L., & Xu, K. (2015). Inactivation of antibiotic resistance genes in municipal wastewater effluent by chlorination and sequential UV/chlorination disinfection, *Science of the Total Environment*,513, 125–132.

Zhang, Y., Marrs, C. F., Simon, C., & Xi, C. (2009). Wastewater treatment contributes to selective increase of antibiotic resistance among Acinetobacter spp. *Science of the Total Environment*, *407*(12), 3702–3706.

Zhuang, Y., Ren, H., Geng, J., & Zhang, Y. (2015). Inactivation of antibiotic resistance genes in municipal wastewater by chlorination, ultraviolet, and ozonation disinfection, *Environmental Science Pollution Research*, *22*, 7037–7044.