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A Sublethal Concentration of Chlorine Induces Antibiotic Resistance in *Salmonella* via Production of Reactive Oxygen Species

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Abstract: Studies have shown that the production of reactive oxygen species (ROS) is triggered by bactericidal antibiotics, which contributes significantly to the killing of bacterial cells and increasing mutations in surviving cells. In this study, we hypothesized that exposure of *Salmonella* to sublethal concentrations of hypochlorite (NaOCl), a commonly used sanitizer in household and food industries increases mutation rates, leading to the development of antibiotic resistance. We found that a sublethal concentration (20 ppm) of NaOCl increased the mutation rates of *S. typhimurium* 14028s significantly ($p < 0.05$), which was prevented by the ROS scavenger thiourea, supporting that the increased mutation was due to NaOCl-triggered ROS production. We further found that the exposure of *S. typhimurium* 14028s to the same sublethal concentration of NaOCl increases resistance to kanamycin among the 3 antibiotics evaluated. The results of this study suggest that when NaOCl applied as a sanitizer fails to kill *Salmonella* due to diluted local concentrations or presence of organic materials, it can cause an adverse outcome of developing antibiotic resistance of the pathogen.

Keywords: *Salmonella*; NaOCl; reactive oxygen species; mutagenesis; antibiotic resistance

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1. Introduction

Salmonella enterica serovar Typhimurium (*S. typhimurium*) is a Gram-negative bacterium that can cause foodborne illness in humans [1,2]. It can cause a wide range of symptoms, including diarrhea, abdominal cramps, fever, and vomiting, resulting in a significant public health burden [3,4]. Consumption of food contaminated with this pathogen, especially uncooked or partially cooked eggs, poultry, meat, and dairy products, is the primary mode of transmission for the disease; however, direct contact with infected animals or their environments can also lead to transmission of the pathogen [4]. In most cases, the resulting condition is ultimately self-limited and usually manifests only mild symptoms [5]. However, under some circumstances, severe cases can occur in vulnerable human populations such as the elderly, young children, and immunocompromised individuals [2,6]. Due to the public health concerns for *S. typhimurium* illnesses, the government regulatory authorities and public health organizations cooperate to detect outbreaks, determine the sources of contamination, and, in turn, implement preventive measures to halt the further spread of the pathogen [7,8]. An additional concern is over the continued development of antibiotic-resistant *Salmonella* strains in both human health and agricultural settings, along

with the genetic mobility of certain antibiotic-resistance genes, thus presenting a potentially higher risk for unsuccessful treatment of *S. typhimurium* infections [9–12].

Numerous studies have been conducted to understand further antibiotic resistance mechanisms associated with various pathogenic *Salmonella* serovars [11,13]. Given the complexity of resistance development, further understanding of the various potential mechanisms requires experiments that examine a multitude of environmental and other external factors that may contribute to the overall level of antibiotic resistance in *Salmonella*. Among these potential factors are reactive oxygen species (ROS), which are well-known chemically reactive molecules formed as a natural result of metabolic processes inside living cells. Superoxide radicals (O_2^-), hydrogen peroxide radicals (H_2O_2), and hydroxyl radicals (OH•) are examples of these molecules. ROS can damage the cellular structures of bacteria, including lipids, proteins, and nucleic acids [14,15]. ROS also acts as a signaling molecule for critical physiological processes such as apoptosis, cell differentiation, and immune system responses [14,15]. It is possible that exposure to ROS can, in turn, lead to the induction of mutations that result in the development of antibiotic resistance in bacterial pathogens such as *S. typhimurium* [14,16]. At the same time, antioxidant defense systems are known to maintain strict control of the amount of ROS internally generated in cells, thus allowing for a precise equilibrium between the beneficial and harmful effects of ROS [17].

Recent research has demonstrated that ROS leads to genetic alterations, which could be responsible for developing unique characteristics such as antibiotic resistance [18]. When ROS-mediated mutagenesis occurs, DNA bases are converted to an oxidized form, resulting in nucleotide disincorporation and structural alterations throughout DNA replication and repair [19,20]. In addition, Dwyer and colleagues demonstrated that ROS-inducing genes may be transferred to *S. typhimurium* and become integrated into its genome to confer antibiotic resistance [18]. Additionally, some studies have reported specific ROS molecules, such as superoxide anion and hydrogen peroxide compounds, that can exert mutagenic effects in bacteria through various mechanisms [21,22]. The respective susceptibility of a pathogen to oxidative stress and its subsequent ability to repair ROS-induced damage after exposure is essential to its survival under various circumstances and environmental conditions, including those occurring in the host [23].

The dynamic interplay between exposure to ROS molecules, mutagenesis, and the subsequent development of antibiotic resistance is a critical aspect of bacterial adaptation. It particularly concerns pathogens such as *S. typhimurium* [24]. Understanding the mechanisms involved is an important step in developing interventions or other mitigation steps to limit the dissemination of antibiotic-resistant strains of *Salmonella* serovars that could present increased public health risks.

Recently, we have shown that ROS production is a major component of the bactericidal activity of NaOCl against various serotypes of *Salmonella enterica* (publication in progress). It was also previously shown that ROS production induced by sublethal concentrations of bactericidal antibiotics increased mutations in *E. coli* through ROS production, which also increased resistance to multiple antibiotics [25]. Therefore, this study was conducted to test the hypothesis that sublethal concentrations of NaOCl can also increase mutation rates, which in turn facilitates the development of antibiotic resistance.

2. Material and Methods

2.1. Microorganisms, Media, and Growth Conditions

Wild type *Salmonella enterica* Typhimurium 14028s (*S. typhimurium* 14028s), obtained from BEI Resources (10801 University Boulevard, Manassas, VA, USA), was used as the model strain for this study. *S. typhimurium* 14028s was stored frozen at −80 °C in Luria Bertani (LB) medium (Fisher Scientific; Waltham, MA, USA) with 15% (*v*/*v*) glycerol. The Institutional Biosafety Committee (IBC) at the University of Arkansas, Fayetteville, AR, approved all laboratory experiments involving this pathogen (Biosafety Level 2). An overnight culture of *S. typhimurium* 14028s in 10 mL LB medium was centrifuged at 2236× *g*

for 10 min, and the resulting supernatant was subsequently discarded. The pellet was re-suspended in 10 mL of M9 medium with 1% glucose as a carbon source from TEKNOVA (2451 Bert Drive, Hollister, CA, USA). The resulting cell suspension was transferred to a 15 mL tube and vortexed to ensure a homogenous cell suspension. A time-zero (T_0) control was established before adding the respective treatments. For the antibiotic resistance experiment, we used chloramphenicol (Cm) (Calbiochem, 10394 Pacific Center Court, San Diego, CA, USA), kanamycin (Kan) (Shelton Scientific, Inc., 230 Long Hill Cross Rd, Shelton, CT, USA), and ampicillin (Amp) (VWR Chemicals, LLC., 6351 Inducon Drive East, Sanborn, NY, USA).

2.2. Rifampicin-Based Selection Assay

Rifampicin was chosen for its role as a selective agent in mutation rate assay, as described previously [26,27]. While rifampicin inhibits RNA polymerase activity, it served as a marker for identifying mutants with alterations in the RNA polymerase gene (*rpoB*), conferring resistance to rifampicin. This approach allows for quantifying mutation frequency in bacterial populations exposed to a sublethal concentration of NaOCl. Twenty ppm of NaOCl was used as the sublethal concentration in the assay, as our preliminary study showed it induced a 50% reduction in bacterial survival [25]. An overnight culture of *S. typhimurium* 14028s was diluted and treated with none (negative control), 20 ppm NaOCl, 20 ppm NaOCl plus 150 mM thiourea or 1 mM H_2O_2. Thiourea, ROS scavenger, was used at the concentration of 150 mM to eliminate ROS produced by NaOCl, as supported by previous studies [28–31]. H_2O_2 was used as a positive control to induce oxidative stress. After 24 h of treatment, cultures were plated on LB agar plates (no antibiotics) to determine colony-forming units per milliliter (CFU/mL) of total viable *Salmonella* cells and on LB agar plates containing rifampicin to select rifampicin-resistant mutants. Colonies were enumerated at 24 and 48 h time points, and the mutation rates were estimated using the maximum likelihood and small-sample (MSS) method [26,32]. The resulting fold change in mutation rate was calculated relative to the untreated negative control.

2.3. Development of Antibiotic Resistance

The next aim was to determine the effect of ROS production in *S. typhimurium* 14028s induced by exposure to NaOCl on the development of resistance to antibiotics. NaOCl at 20 ppm was used to induce ROS production. An overnight culture of *S. typhimurium* 14028s was exposed to 20 ppm NaOCl or PBS (no exposure control) for 12 h and then 10-fold serial dilutions were plated on LB agar plates to determine the initial populations. The same cultures were also plated on LB agar plates supplemented with Amp (30 μg/mL), Cm (30 μg/mL) or Kan (50 μg/mL) to enumerate the resistant colonies to respective antibiotics. All plates were incubated overnight at 37 °C. The colonies were counted and used to calculate the survival % for each group.

2.4. Statistical Analysis

Each treatment group included three biological replicates to assess statistical significance. The differences between the mean mutation rates or the survival % were analyzed by one-way analysis of variance (ANOVA). A p-value of less than 0.05 was considered statistically significant. All analysis was performed using JMP (JMP®, Version JMP pro-17, SAS Institute Inc., Cary, NC, 1989–2023, USA).

3. Results

3.1. NaOCl Exposure and Mutation Rates

We hypothesized that a sublethal concentration of NaOCl induces ROS production, leading to an increase in the mutation rate. As shown in Figure 1, *S. typhimurium* 14028s showed the significant increase in the mutation rates (over 30-fold) after exposure to NaOCl (20 ppm) as compared to the negative control with no exposure ($p < 0.0006$). As expected,

the addition of thiourea prevented the increase in the mutation rate by NaOCl, suggesting that ROS production by NaOCl was responsible for the increased mutation rate (Figure 1).

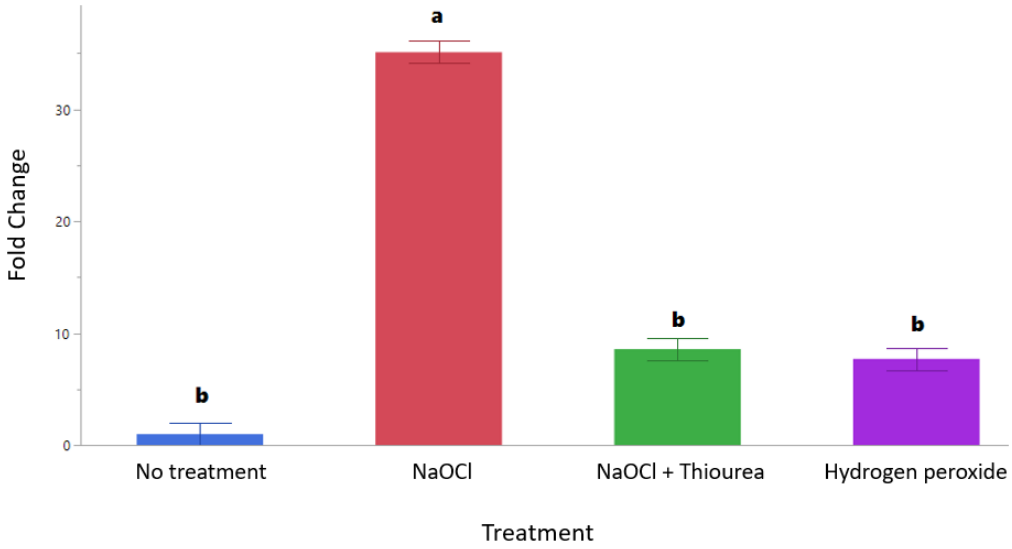

Figure 1. The mutation rates of *S. typhimurium* 14028s after exposure to a sublethal concentration of NaOCl. *S. typhimurium* 14028s was exposed to NaOCl (20 ppm) or NaOCl (20 ppm) plus thiourea (150 mM) before determination of the mutation rate using rifampicin. The same procedure was repeated with no exposure (no treatment; negative control) or exposure to H_2O_2 (1 mM; positive control). Different letters denote statistical differences ($p < 0.0006$).

3.2. NaOCl Exposure and Resistance to Other Antibiotics

After we observed that the exposure to NaOCl significantly increased the mutation rate, we hypothesized that the ROS produced by NaOCl might also increase the resistance of *S. typhimurium* 14028s to other antibiotics. Therefore, we selected 3 antibiotics, ampicillin (Amp), chloramphenicol (Cm), and kanamycin (Km) for this evaluation. For each antibiotic tested, the group without exposure to NaOCl was used as the respective negative control for comparison. As shown Figure 2, we found a significant increase in antibiotic resistant colonies for Km ($p < 0.0001$), reaching 45.8% survival after exposure to NaOCl. On the contrary, there was no significant change for the other two antibiotics, Amp and Cm.

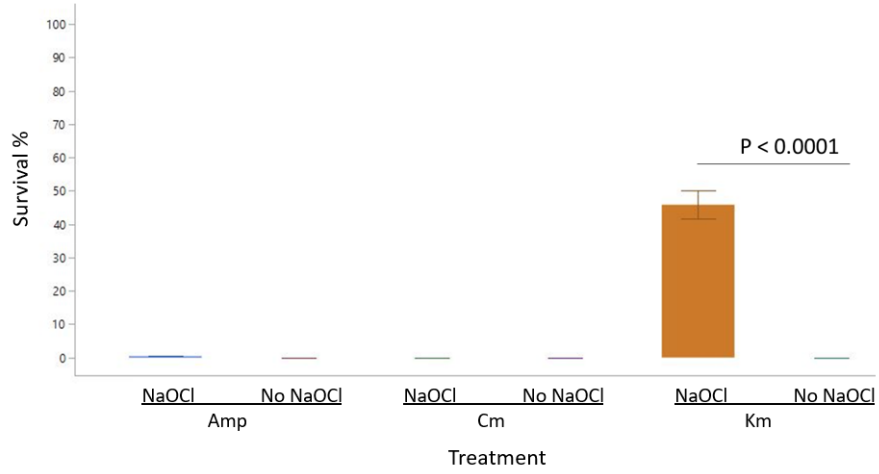

Figure 2. Development of antibiotic resistance after exposure to a sublethal concentration of NaOCl. *S. typhimurium* 14028s was exposed to NaOCl (20 ppm) or none (negative control) before determination of the resistance to different antibiotics (Amp, Cm or Km) as the % of the surviving cells.

4. Discussion

Foodborne *Salmonella* serovars continue to contribute to ongoing public health problems and can become an even greater concern if they acquire resistance to antibiotics [9–12]. There are a number of ways in which *Salmonella* becomes resistant to various antibiotics. This can occur for a variety of reasons, including exposure to external factors and intracellular responses in pathogens. Depending on the concentration, exposure to antibiotic compounds can certainly contribute to these responses by *Salmonella* and other pathogens. Among the metabolic processes that can potentially interface with antibiotic exposure are ROS molecules. ROS-induced mutagenesis plays a role in adaptive evolution and, notably, in the development of antibiotic resistance, where mutations in genes associated with antibiotic targets or the resulting expression of resistance mechanisms can provide a selective advantage to bacteria exposed to antibiotics [18,32]. This is particularly important for pathogens such as *Salmonella*. If such mechanisms occur in *Salmonella* serovars, this represents an additional public health concern and could increase potential risk for medical treatment. Consequently, identifying potential linkages between antibiotic exposure in *Salmonella* and subsequent ROS production that result in increased antibiotic resistance should be examined. This research aimed to further investigate the potential connection between ROS-induced mutagenesis and the evolution of antibiotic resistance in *S. typhimurium* 14028s, providing additional insight into the factors associated with the development of antibiotic resistance and, subsequently, bacterial adaptation.

A previous study using *E. coli* showed that the production of ROS is a common mechanism among all 3 major classes of bactericidal antibiotics whereby the antibiotics kill bacterial cells, regardless of their distinct targets for initial interactions [25,27]. The subsequent study by the same group also demonstrated that low level of ROS production induced by sublethal levels of the bactericidal antibiotics led to development of multi-drug resistance in *E. coli* [25,27]. The result is also well supported by the fact that certain ROS, such as hydroxyl radicals, can directly damage DNA and accumulate mutations [25,27] An erroneous DNA repair mechanism can lead to genetic alterations such as rifampin resistance, which can occur because of a mutation in the *rpoB* gene (a subunit of RNA polymerase) [33]. Furthermore, it has been demonstrated that ROS production plays a central role in various process of cell death, including both eukaryotic and prokaryotic cells [34].

In our recent study prompted by the common role of ROS production in cell death, we showed the result that ROS production plays a key role in killing of various serotypes of *S. enterica* by NaOCl (publication in progress), which are widely used as sanitizers in both household and food industries. Therefore, we hypothesized that sublethal levels of NaOCl can also increase mutations in *S. typhimurium* through ROS production.

The result of this study demonstrated that when *S. typhimurium* 14028s was exposed to a sublethal concentration (20 ppm) of NaOCl, it increased the mutation rate significantly. The observation that the increase in mutation rate was prevented when thiourea, the ROS scavenger, was added further supports our conclusion. When the number of colonies resistant to different antibiotics was determined with and without exposure to 20 ppm NaOCl, there was a dramatic increase in antibiotic resistant colonies for kanamycin. It remains unclear why *S. typhimurium* 14028s showed increased resistance to kanamycin, but not to the other two antibiotics tested, which warrants further studies. Other studies have suggested interactive relationships between ROS and various antibiotics [35–37].

These results could be further validated by sequencing of the target genes and/or whole-genome sequencing to identify the genes associated with developing antibiotic resistance. It would also be important to examine if other strains of *S. typhimurium* or other serotypes of *S. enterica* can elicit similar responses by increasing mutation rate upon exposure to sublethal levels of NaOCl. If there is variability in responses to NaOCl, strain and/or serovar genetic differences could account for some of that variability. Further investigations are needed in order to track down the underlying mechanism(s) whereby NaOCl can induce mutations in certain genes and to determine how this impacts the transmittance

of antibiotic resistance among bacterial populations. More specifically, linking certain genes with NaOCl exposure may help to further identify the functional properties associated with these genes that could be related to the direct impact of NaOCl on pathogens such as *Salmonella*. In addition, identifying other potential factors that may play a role, in conjunction with NaOCl, the induction of mutation responses needs to be prioritized in future studies. Given the importance of chlorine-based compounds as sanitizing and disinfecting agents, this would be important to understanding and potentially predicting their efficacy with respect to different commercial applications.

5. Conclusions

Antibiotic resistance continues to be a global concern for public health. This holds true for many pathogens, including foodborne pathogens that tend to contaminate a wide range of foods during their production cycle. Along with animal-based food products, this contamination includes not only vegetables, nuts, and fruits but also other non-meat food products in the food chain [38]. This is further complicated by the potential for cross-contamination among different foods and ingredients and from the environment during food or meat processing and/or meal preparation. Regardless of the pathogen's origin, foodborne pathogens that encounter food and, in turn, result in contamination can lead to illnesses in susceptible humans. If these foodborne pathogens are antibiotic-resistant, they can become recalcitrant to prescribed antibiotic therapies used to treat the disease. This holds for *Salmonella* serovars such as *S. typhimurium*, as well other *Salmonella* serovars that can cause foodborne salmonellosis, and, depending on their dissemination patterns and whether they become antibiotic resistant, they could not only compromise medically prescribed antibiotic treatments but also impact efforts to limit widespread outbreaks of foodborne disease.

Given the widespread use of NaOCl or other chlorine-based compounds as sanitizing and disinfecting agents, the result of this study has an important implication concerning public safety. Although effective, when NaOCl fails to kill pathogens, such as *S. typhimurium*, due to the presence of organic materials or diluted local concentrations, it can develop antibiotic resistance in the surviving subpopulations of the pathogens. A key question is determining the factors that may impact the introduction of antibiotic resistance in foodborne pathogens such as *Salmonella* and lead to the evolution and emergence of multiple antibiotic resistances. While the current study represented only a limited assessment of antibiotic resistance with only one strain of *S. typhimurium*, it does further support the concern that resistant strains of *Salmonella* present additional challenges for clinical treatments by limiting the effective range of antibiotics that can be successively administered. Environmental stimuli that induce mutagenesis resulting in multi-drug resistance, whether from environmental conditions or host-derived, can introduce a certain level of uncertainty in predicting a successful antibiotic treatment. This may include not only the choice of antibiotics but also which combinations to use. Equally important is the impact of antimicrobials on subsequent antibiotic resistance in *Salmonella* and other foodborne pathogens.

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