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# Research Article
# Rugose Morphotype of *Salmonella enterica* Serovar Typhimurium ATCC14028 Exhibits Chlorine Resistance and Strong Biofilm Forming Ability

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## Abstract

**Background and Objective:** Antibiotic resistance is a major global public health problem and studies have shown that when bacteria adapt to one stress, they can offer that protection to resist other stresses. Therefore, the aim of this study was to examine the ability of *Salmonella* to adapt to chlorine. **Methodology:** *Salmonella* Typhimurium (American Type Culture Collection, ATCC 14028) was tested for its ability to adapt to increasing increment of chlorine starting at 125 ppm in tryptic soy broth (TSB). *Salmonella* Typhimurium demonstrated an acquired tolerance to chlorine in TSB with adapted cells growing in concentrations up to 600 ppm whereas the non-adapted cells did not grow beyond 500 ppm. **Results:** After 4 days of incubation, *S. typhimurium* exposed to sublethal chlorine concentrations displayed a distinct rugose and smooth morphology on tryptic soy agar (TSA) plates incubated at 37°C. The rugose, in contrast to smooth morphology (both adapted and control), showed the ability to form very strong biofilms ($p<0.05$) in polystyrene microtiter plates at room temperature and 37°C. The antibiotic susceptibility patterns of adapted (rugose and smooth) and control cells were tested using different antibiotics according to the Clinical and Laboratory Standards Institutes (CLSI) guideline. There was only slight difference observed in antibiotics resistance of either adapted cell type as compared to control. **Conclusion:** The incorrect application of chlorine during cleaning and sanitation could select for adapted *Salmonella* cells, which may attach strongly to plastic surfaces in a processing facility.

Key words: *Salmonella*, chlorine, adapted cells, rugose, biofilms

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Data Availability: All relevant data are within the paper and its supporting information files.

## INTRODUCTION

Around the world, nontyphoidal *Salmonella* is a major foodborne pathogen causing numerous reported cases of foodborne infections[1,2]. It was reported to result in over 40,000 cases leading to approximately 500 deaths each year by the Centers for Disease Control and Prevention (CDC)[3]. Gastroenteritis and bacteremia are caused by nontyphoidal *Salmonella*[3]. In the past year, the CDC estimated that nontyphoidal *Salmonella*, which is identified as the leading cause of diarrhea in most parts of the world, caused 94 million cases of gastroenteritis and 115,000 deaths globally[3]. Due to the food safety concerns imposed by *Salmonella* and other foodborne pathogens, the USDA-FSIS has a list of substances (antimicrobials) that are approved to be used during the production of poultry, meat and egg products (FSIS directive 7120.1)[4]. The poultry industry uses the antimicrobials directly in the chiller water and by spraying or dipping poultry products. In addition, a thorough cleaning, disinfecting and sanitization program for contact surfaces, equipment and poultry processing facility are incorporated into good manufacturing practices (GMP) by the industry.

However, bacteria can still aggregate on surfaces to form biofilms, which are a considerable food safety concern. It was reported that about 80% of all the infections caused by microorganisms, including foodborne illnesses are caused by microorganisms that are present as a biofilm[5,6]. A biofilm is composed of several microorganisms that interact with each other and become attached to an exposed surface by a matrix produced by the organisms[7,8]. The film produced by the microbes is covered by an exopolysaccharide layer consisting of cellulose and curli fimbriae[9,10]. Bagge-Ravn *et al*.[11] suggests that curli fimbriae are the main protein nutrient of the biofilm matrix. They function by aiding in the aggregation and adhesion of bacteria cells to surfaces thus forming a full mature biofilm[10]. Several studies have reported the microbial composition of biofilms that can include species such as *Vibrio* spp., *Listeria monocytogenes*, *E. coli* and other spoilage microbes in different food processing environments[12,13]. Some of these studies observed that the pathogens in biofilms could be isolated even after cleaning and sanitization[12].

In food processing, the ability of foodborne pathogens to attach to and subsequently form a biofilm on food contact surfaces may constitute a potential source of post-processing cross contamination of products[14]. Chemical treatment like chlorine and chlorine-based compounds, quaternary ammonium compounds, organic acids (peroxyacetic and acetic acid), hydrogen peroxide and iodine compounds are commonly used to remove any attached cells[15]. However, several studies have shown that bacteria in biofilms are more resistant to antimicrobial treatment and sanitation procedures when compared to planktonic cells[10,15-20].

Among the approved chemical sanitizers for food-contact surfaces, chlorine and choline-based compounds are the most widely utilized. Chlorine is not costly and provides a broad-spectrum bactericidal activity[21,22]. The recommended level of chlorine for sanitation purposes is 200 ppm and it is most effective at a pH of 6.5 or below[23,24]. However, the use of antimicrobials including chlorine at a sublethal concentration may constitute a crucial public health risk.

The increase in pathogenic bacterial resistance towards antibiotics and antimicrobials is a persistent public health challenge all over the world[25,26]. Exposure of food borne pathogens to sublethal concentrations of sanitizing agents like chlorine may create a potential challenge to the bacteria[26]. The possibility of such pathogenic bacteria adapting to the stress and using the adaptive resistance to induce cross-resistance to antibiotics has been elucidated by others[27-30]. The fact that chlorine is the most common sanitizing agent used in food processing and bacteria can survive when chlorine is used at a sublethal level, is concerning. However, the greater concern is when the exposed pathogens confer cross-adaptation to clinically important antibiotics.

In this study, exposure of *Salmonella* enterica serovar Typhimurium to sublethal concentrations of chlorine caused a morphological change to the rugose variant of *Salmonella*. Anriany *et al*.[31] observed the rugose phenotype in *S. typhimurium* DT104 on TSA after three days of incubation at 25°C. It was defined as a corrugated colony morphology associated with the formation of exopolysaccharide (EPS) and cell aggregation[32]. Rugosity usually develops in response to unfavorable conditions such as stress and starvation[31-33-36]. Studies have shown that the rugose variant in *V. cholerae* displayed increased resistance to chlorine treatment, which may have aided in the survival of *Vibrio* in various food borne outbreaks[31,35-37]. Nevertheless, the ability of rugose cells to adapt to chlorine treatment has not been well reported in *S. typhimurium* ATCC14028.

Therefore, the main aim of this study was to determine homologous stress adaptation by measuring the change in the minimum inhibitory concentration (MIC) both pre and post exposure to chlorine. When this aim was achieved, the

difference in biofilm forming ability of stress adapted compared to non-adapted cells were determined. Also, the possibility of cross-adaptation to different antibiotics was assessed.

## MATERIALS AND METHODS

**Inoculum preparation:** One loop full of frozen *Salmonella* Typhimurium (American Type Culture Collection, ATCC 14028) culture was obtained and streaked on a tryptic soy agar plate (TSA, Sigma-Aldrich Co., St. Louis, MO, USA). The plate was subsequently incubated in a refrigerated incubator (Thermo Fisher Scientific, Waltham, MA) at 37°C for 24 h. The cells were maintained through monthly transfer on TSA slants that were stored at 4±1°C. The broth culture was prepared by transferring a single colony from a TSA plate into sterile 10 mL tryptic soy broth (TSB, Sigma-Aldrich Co., St. Louis, MO), which was incubated at 37°C for 24 h to achieve a total plate count of approximately $10^9$ CFU $mL^{-1}$. Cells were harvested by centrifugation (Eppendorf Biotech company Hamburg, Germany) at 5500 rpm for 10 min at 4°C. The supernatant was discarded and the pellet was re-suspended in fresh 10 mL sterile TSB to make a stock culture of ~$10^9$ CFU $mL^{-1}$.

**Sanitizing agent:** Chlorine, in the form of sodium hypochlorite, containing 5% available chlorine (ACROS Organics, New Jersey USA), was used in this study. The concentration of free chlorine was validated using the HACH (chlorine test kit) Pocket Colorimeter (HACH Company, Loveland, CO, USA) according to the manufacturer's instructions.

**Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) assay:** The MIC and MBC of chlorine were determined for *Salmonella* Typhimurium with a 96-well polystyrene microtiter plate (Thermo Fisher Scientific, Waltham, MA, USA) using the broth microdilution method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines with minimal modifications[38]. A volume of 200 µL, twice the amount of sodium hypochlorite in TSB was added to the first well of the microtiter plate as an initial concentration. Then 100 µL of sterile TSB was added to all other wells in the same row. The sanitizing agent was diluted two-fold by transferring 100 µL of sodium hypochlorite from the first well through the last well and the excess was discarded. After diluting the sanitizing agent, 100 µL of the inoculum prepared by serial dilution of the stock culture (final concentration of ~$10^6$ CFU $mL^{-1}$) was added to each well. A positive control (i.e., 100 µL TSB+100 µL inoculum) and negative control (i.e., 200 µL of TSB without inoculum) were maintained throughout the experiment. Bacterial growth for each well was determined by turbidity after incubation at 37°C for 24 h. The MIC was determined to be the lowest concentration of the sanitizing agent in which there was no bacterial growth. The sanitizer was tested in all the 96-wells for *S. typhimurium*. To determine the MBC of sodium hypochlorite against *S. typhimurium*, the wells showing no visible bacterial growth after 24 h of incubation at 37°C were selected. An aliquot of 50 µL from the clear wells was spread plated on TSA plates and the plates were incubated at 37°C for 24 h. The highest dilution of chlorine that showed no bacterial growth on TSA plates after incubation was then considered to be the MBC.

**Exposure to increasing concentrations of chlorine:** For the stress adaptation study, the overnight stock culture was prepared to a final concentration of ~$10^9$ CFU $mL^{-1}$. An aliquot of 100 µL of the stock culture was added to 9.9 mL TSB containing a starting concentration of 125 ppm chlorine in a (15mL) sterile polypropylene flat cap tube (Fisher Scientific, Fair Lawn, N J) to make a final inoculum concentration of ~$10^7$ CFU $mL^{-1}$. The tube was incubated at 37°C for 24 h, bacterial growth in the tube was observed for turbidity and when growth was observed, 100 µL of the suspension was aseptically transferred into a sterile tube, which contained 9.9 mL TSB with the next highest concentration of chlorine. The suspension was then diluted and plated on TSA and incubated overnight at 37°C to observe *Salmonella* growth. The procedure continued with daily increases (25 ppm) of chlorine until a concentration of chlorine was reached where there was no visible bacterial growth observed after incubation at 37°C. This required a total of 18 days. The suspension from the last tube with visible growth was plated on TSA plates without chlorine and the cells obtained after incubation at 37°C were considered to be adapted *S. typhimurium* cells. The agar plates were kept at 4±1°C with weekly transfer. Non-exposed cells were grown in TSB without chlorine and were subsequently diluted and plated on TSA plates to serve as the control. The stress adaptation study was replicated three times over the course of 3 months.

**Stability of adapted cells to homologous stress:** To determine the stability of the adapted cells, the MIC and MBC of chlorine was determined against the adapted cells of *S. typhimurium* ATCC14028 with disposable borosilicate glass round bottom culture tubes (Fisher Scientific, Fair Lawn,

NJ) using the broth macrodilution method in accordance with the CLSI guidelines[39]. A colony of adapted *Salmonella* cells was inoculated into glass tubes containing TSB with chlorine at (i) A concentration below MIC, (ii) A concentration equivalent to MIC and (iii) Two concentrations that are 50 and 100 ppm, respectively above MIC. Non-adapted (control) *Salmonella* cells were also tested at the same concentrations. The adapted and control cells were tested after storage on TSA plates without chlorine. The experiment was replicated three times in duplicate glass tubes. The glass tube showing no turbidity was considered to be the MIC of the adapted and control cells. For MBC determination, the glass tube showing no bacterial growth after incubation at 37°C for 24 h was selected. An aliquot of 100 µL from the clear tube was spread plated on TSA plates and was incubated for approximately 24 h at 37°C. The lowest concentration of chlorine that showed no visible colonies on TSA plates after incubation was considered the MBC of adapted and control cells.

**Biofilm formation study:** The biofilm forming ability of *S. typhimurium* ATCC 14028 cells was performed using a previously described method[40]. Adapted cells were grown at 37°C for 24 h and appropriate dilutions in TSB were prepared to obtain a final inoculum concentration of ~$10^6$ CFU $mL^{-1}$. TSB containing 550 ppm chlorine (the highest concentration that supports bacterial growth) was used for the growth of previously adapted cells and TSB without chlorine was used for the control cells. The microtiter plate was prepared by adding 200 µL of the culture and was then incubated at both room temperature and 37°C for 48 h. After incubation, the inoculum was completely removed from each well by aspiration and the wells were washed five times with sterile distilled water in order to remove any loosely attached bacteria. The plate was allowed to air dry for 45 min. Afterwards, 200 µL of crystal violet solution (0.41% w/v dye) was added to each well and incubated at room temperature for 45 min. Following incubation, the crystal violet solution was completely removed from the wells by aspiration and the wells were washed five additional times with sterile distilled water. The plate was air dried for 45 min and then 200 µL of 95% ethanol was added to each well and the content of the wells was mixed. The biofilm formation in the well was measured by taking optical density readings at 600 nm ($OD_{600}$) using a micro-quant microplate spectrophotometer (BioTek Instruments, Winooski, VT).

**Enumeration of attached cells:** The biofilm formation of chlorine adapted and control cells were also determined by enumerating the number of strongly attached cells on a plastic surface using the 24-well polystyrene plate (Celltreat Scientific Product, Pepperell, MA). Similar to the crystal violet assay, ~$10^6$ CFU $mL^{-1}$ of adapted and control cells were obtained and 200 µL of the inoculum was put into each well. The plates were incubated at room temperature and 37°C for 48 h. Afterwards, the wells were emptied by removing the inoculum and each well was washed with sterile distilled water three times to remove any loosely attached cells. The strongly attached cells in each well was subsequently scraped into 0.1% peptone water. The suspension was then vortexed for 2 min, subjected to a 10-fold serial dilution and a volume of 100 µL was spread plated on TSA plates. The number of cells after incubation at 37°C for 24 h were enumerated.

**Antibiotic susceptibility testing:** The chlorine stressed and control cells were screened for susceptibility to a range of antibiotics in Mueller-Hinton broth and on Mueller-Hinton agar (MHB, MHA, Oxoid Co. Canada) by the disk diffusion and MIC broth microdilution method as described in the CLSI guidelines[38]. The antibiotic discs used were sulfamethoxazole/trimethoprim (SXT, 25 µg), gentamicin (GN, 10 µg), streptomycin (S, 10 µg), amoxicillin/clavulanic acid (AMC, 30 µg), nalidixic acid (NA, 30 µg), ciprofloxacin (CIP, 5 µg), ceftriaxone (CTX, 30 µg) and ampicillin (AMP, 10 µg). The zones of inhibition were measured and recorded as susceptible, intermediate and resistant according to the CLSI guidelines[38]. The broth microdilution for the antibiotics was performed in a 96-well polystyrene microtiter plate. Each panel contains approximately 6 dilutions using the MIC breakpoints recommended by the CLSI guidelines[38]. One well represented the positive control (i.e., broth and inoculum) and one served as the negative control (broth only). The results were observed and recorded as the least concentration of antibiotics that inhibit the growth of *Salmonella* either as susceptible, intermediate or resistant according to the CLSI guidelines. All combinations of *Salmonella* cells (adapted and control) with antibiotics were conducted on separate days.

**Statistical analysis:** All experiments were replicated three times sequentially. Analysis of variance (ANOVA) in the General Linear Model (GLM) of SAS v. 9.4, SAS Institute[41] was used to analyze the data. The means were separated by Fisher's Least Significant Difference test. The treatments and controls were considered to be significant when $p \leq 0.05$.

## RESULTS

**Observations during stress adaptation study:** When *S. typhimurium* reached a 200 ppm concentration of chlorine in TSB which was exactly 4 days of incubation from the start of the stress adaptation, a mixed colony with two different morphologies were observed on TSA plates. One morphology appeared to be rough and dry, it is referred to as the "rugose" morphotype of *S. typhimurium*. The rugose and normal smooth *Salmonella* morphotypes continued to grow together on each day of transfer until the concentration of chlorine in TSB reached 550 ppm. This concentration was identified as the highest chlorine concentration that supports bacterial growth. The rugose and smooth *Salmonella* cells harvested at this concentration were considered to be the adapted *Salmonella* cells. In suspension, the rugose morphotype remained as a cell aggregate, whereas the smooth morphotype was dispersed in the broth making a homogenous solution.

**Adaptation to chorine stress:** The MIC of chlorine against *S. typhimurium* ATCC 14028 prior to exposure to sublethal concentrations of chlorine was observed to be 400 ppm using the broth microdilution method. When the glass-tube broth macro-dilution method was used, 500 ppm was determined to be the MIC. The MBC was observed to be 500 ppm and this value was the same for all replications (Table 1). After several passages through increasing concentrations of chlorine, the maximum concentration of chlorine that allowed growth after 18 days of incubation at 37°C was 550 ppm. This showed that the adapted cells were able to grow in concentrations of chlorine that are 1.38 times higher than the control cells.

**Homologous stress adaptation:** The MIC and MBC of adapted rugose and smooth *Salmonella* cells were determined to maintain their stability to chlorine. The adapted *Salmonella* cells (rugose and smooth) were stored on TSA plates without chlorine. Single colonies were transferred weekly to a fresh chlorine-free TSA plates to ensure storage. The adapted rugose and smooth cells were able to grow at 450, 500, 550 and 600 ppm chlorine concentrations whereas, the non-adapted cells did not grow beyond 500 ppm. Thus, the MIC changed from 500 ppm before adaptation to 650 ppm after adaptation for the chlorine adapted *Salmonella* cells, which means homologous stability lasted even without the presence of chlorine (Table 2).

Table 1: Minimum Inhibitory Concentrations (MICs) and Minimum Bactericidal Concentrations (MBCs) of chlorine (ppm) for *S. typhimurium* (ATCC 14028) a before adaptation

| Dilution method | MIC (ppm) | MBC (ppm) |
|---|---|---|
| Micro | 400 | 500 |
| Macro | 500 | 500 |

[a] *Salmonella* typhimurium culture prior to chlorine exposure. Data represent the average of 3 replicates

Table 2: Minimum Inhibitory Concentrations (MICs) of chlorine (ppm) for *S. typhimurium* (ATCC 14028) after stress adaptation

| *S. typhimurium* morphotypes | [a]Chlorine concentration (ppm) |
|---|---|
| Adapted rugose | 650 |
| Adapted smooth | 650 |
| Control | 550 |

[a] *Salmonella* cultures after exposure to increasing sublethal concentrations of chlorine, control represent unexposed *Salmonella* culture. Data represent the average of 3 replicates

**Biofilm formation on plastic surface:** The adapted and control *S. typhimurium* cells were tested for their ability to form biofilms on a polystyrene plastic surface (using 96-well polystyrene microtiter plate). The adapted *Salmonella* cells were cultured in TSB containing 550 ppm chlorine. The optical density (OD) reading at 600 nm of the adapted (rugose and smooth) and control cells was observed. At 37°C, the biofilm forming ability of all the *S. typhimurium* morphotypes tested (adapted and control) was significantly different ($p<0.0001$) compared to the negative control (Fig. 1a). The adapted rugose cells formed the strongest biofilms on the plastic surface ($p<0.0001$) with the highest OD600 values averaging 3.4 and 3.64 at 37°C and room temperature, respectively. Whereas, the adapted smooth cells were a better biofilm former compared to the control cells, with both having an average $OD_{600}$ values of 0.8 and 0.47 at 37°C and 0.63 and 0.48 at room temperature. As expected, the negative control, which is TSB without bacteria or chlorine had the lowest $OD_{600}$ value averaging 0.14 and 0.15 at 37°C and room temperature, respectively. Although, adapted rugose cells formed better biofilms at room temperature ($p<0.0001$), there was no significant differences observed in the biofilm forming ability of the adapted smooth as compared to the non-adapted control *Salmonella* cells (Fig. 1b). The strongly attached cells in the plastic plate were also enumerated. The results demonstrated that a significant difference ($p<0.05$) could be observed between all the cell types tested at both temperatures. The adapted rugose variant had a cell concentration of 5.25 and 5.3 log CFU $mL^{-1}$ at 37°C and room temperature, respectively. While the adapted smooth and control had a cell concentration of 4.6 and 4.3 log CFU $mL^{-1}$ at

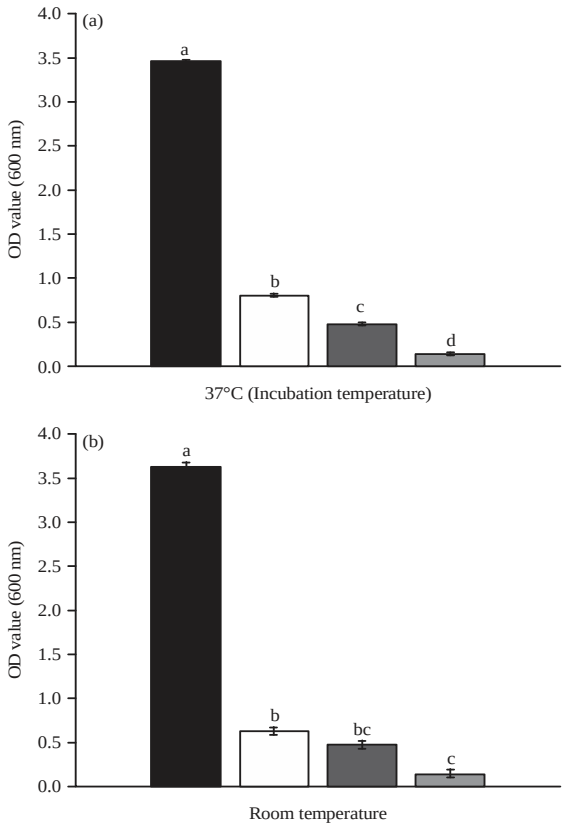


Fig. 1(a-b): (a) Biofilm formation by *S. typhimurium* after 48 h on plastic surface using 96-well polystyrene microtiter plate at 37°C. Means with different superscripts indicate significant differences in the biofilm forming ability of chlorine-adapted and control *S. typhimurium* morphotype ($p<0.0001$, SEM: 0.013, N = 3), (b) Biofilm formation by *S. typhimurium* after 48 h on plastic surface using 96-well polystyrene microtiter plate at room temperature. Means with different superscripts indicate significant differences in the biofilm forming ability of chlorine-adapted and control *S. typhimurium* morphotype ($p<0.0001$, SEM: 0.045, N = 3)

The black bar represents chlorine-adapted rugose morphotype, white bar represents chlorine-adapted smooth morphotype, dark grey bar represents non-exposed positive control and light grey bar represent (broth only) negative control

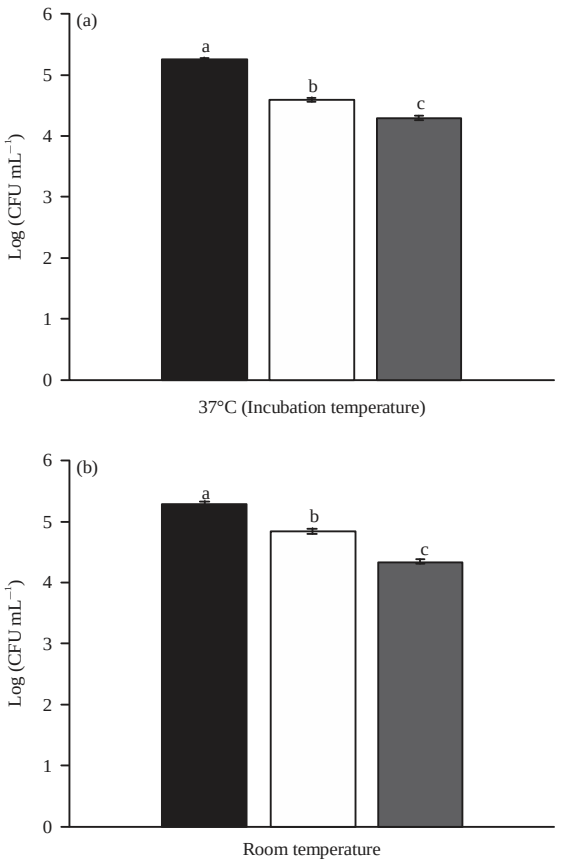


Fig. 2(a-b): (a) Quantification of *S. typhimurium* after 48 h attached to 24-well polystyrene plate at 37°C. Means with different superscripts indicate significant differences in the quantification of the attached chlorine-adapted and control *S. typhimurium* morphotype on the polystyrene plastic plate ($p = 0.0004$, SEM: 0.029, N = 2), (b) Quantification of *S. typhimurium* after 48 h attached to 24-well polystyrene plate at room temperature. Means with different superscripts indicate significant differences in the quantification of the attached chlorine-adapted and control *S. typhimurium* morphotype on the polystyrene plastic plate ($p = 0.0011$, SEM: 0.041, N = 2)

The black bar represents chlorine-adapted rugose morphotype, white bar represents chlorine-adapted smooth morphotype and dark grey bar represents non-exposed control

37°C and 4.85 and 4.35 at room temperature. The rugose cell concentration was more on the plastic surface when compared to the smooth adapted and non-adapted control cells at 37°C and room temperature (Fig. 2a and b).

**Antibiotic cross-adaptation:** The *S. typhimurium* was screened for susceptibility to different antibiotics after exposure to increasing sublethal concentrations of chlorine. The antibiotic resistance patterns of the adapted (rugose and smooth) and control cells are shown in Table 3 and 4. The

Table 3: Antibiotic cross-resistance patterns of adapted and control *S. typhimurium*

| *S. typhimurium* cultures previously adapted to chlorine[b] | Resistance to indicated antibiotics SXT GN S AMC NA CIP CTX AMP |
|---|---|
| Adapted rugose | 20/S 13/I 8/R 21/S 15/I 23/I 22/I 18/S |
| Adapted smooth | 20/S 13/I 8/R 21/S 15/I 25/I 23/I 18/S |
| Control | 20/S 13/I 10/R 21/S 18/I 24/I 23/I 18/S |

[a]SXT: Sulphamethoxazole/trimethoprim (25 µg), GN: Gentamicin (10 µg), S: Streptomycin (10 µg), AMC: Amoxicillin/clavulanic acid (30 µg), NA: Nalidixic acid (30 µg), CIP: Ciprofloxacin (5 µg), CTX: Ceftriaxone (30 µg), AMP: Ampicillin (10 µg). Resistance zones of inhibition (millimeters) are reported as S: Susceptible strain, I: Intermediate susceptible strain, R: Resistant strain. Boldfaced data indicate reduced susceptibility relative to unexposed (control) strains; data not bolded indicate exposed strains with no difference in susceptibility patterns relative to unexposed (control) strains. An increase in resistance was defined as a change in S (before chlorine exposure) to R (after chlorine exposure). [b] For adaptation, cultures were previously exposed to increasing sublethal concentrations of chlorine, control represent unexposed *Salmonella* culture. Data represent the average of 3 replicates

Table 4: Broth microdilution assay of antibiotic cross-resistance patterns of adapted and control *S. typhimurium*

| *S. typhimurium* cultures previously adapted to chlorine[b] | Antibiotic[a] (µg mL$^{-1}$) AMP GN S NA T CIP AMX |
|---|---|
| Adapted rugose | 4/S 4/S 64 16/S 4/S 1/S 32/R |
| Adapted smooth | 2/S 2/S 32 16/S 4/S 1/S 32/R |
| Control | 2/S 2/S 16 8/S 2/S 1/S 16/I |

[a]AMP: Ampicillin, GN: Gentamicin, S: Streptomycin, NA: Nalidixic acid, T: tetracycline, CIP: Ciprofloxacin, AMX: Amoxicillin. Resistance MIC are indicated as S: Susceptible strain, I: Intermediate susceptible strain, R: Resistant strain. Boldfaced data indicate reduced susceptibility relative to unexposed (control) strains; data not bolded indicate exposed strains with no difference in susceptibility patterns relative to unexposed (control) strains. An increase in resistance was defined as a change in S (before chlorine exposure) to R (after chlorine exposure). [b]For adaptation, cultures were previously exposed to increasing sublethal concentrations of chlorine, control represent unexposed *Salmonella* culture. Data represent the average of 2 replicates

results showed that there was no significant differences in the antibiotic susceptibility patterns of the adapted cells when compared to the control cells for any of the antibiotics tested. However, the rugose cells did show a slight reduction ($\leq$2 mm) in susceptibility to streptomycin, nalidixic acid, ciprofloxacin and ceftriaxone when compared to smooth and control. A change in MIC (antibiotic susceptibility) was observed for the adapted cells (rugose and smooth), which exhibited resistance to amoxicillin. In addition, a slight increase in MIC was observed for rugose cells against all the antibiotics tested except ciprofloxacin compared to smooth (adapted and control).

## DISCUSSION

In the current study, the rugose variant of *S. typhimurium* was observed in response to the exposure of the smooth variant to sublethal concentrations of chlorine starting at 200 ppm at 37°C. Similarly, the rugose phenotype has been observed in both *Vibrio cholerae* (O1 and non-O1 groups) and *Salmonella enterica* serovar Typhimurium DT104 and non-DT104[31,32,42,43]. However, in the case of *Vibrio cholerae*, the rugose variant was observed after several passages of the smooth variant in alkaline peptone water at 37°C and in response to nutrient starvation when grown at 16°C for several days[32,35,36]. In *Salmonella* Typhimurium DT104, the rugose phenotype was observed after 4 days of extended incubation of the smooth colony at 19-28°C on TSA plates[31]. Contrary to the observations in the current study, Anriany *et al*.[31] suggested that the rugose phenotype was either temperature or media dependent because it was not identified upon incubation at 37°C and it required more incubation time to grow on brilliant green agar (BGA). In the current study, the rugose morphotype was observed on brilliant green sulfa agar (BGSA) but could not be observed on XLT4 agar. There was no difference in the appearance of rugose and smooth *Salmonella* morphotype on (XLT4) agar plates, which is in agreement with the observations of Anriany *et al*.[31]. The findings in this study suggest that chlorine is a stressor that induce the formation of the rugose morphotype in *S. typhimurium* ATCC14028.

This study attempted to investigate the significant effect of *S. typhimurium* adaptation to an antimicrobial that has been used in food processing for an extended period, either directly or as a sanitizing agent on food-contact surfaces. Chlorine in the form of sodium hypochlorite is reported to be more frequently used by food processors[44]. There are previous reports on the adaptation of pathogenic microorganisms to food-grade biocides and antimicrobials used in food production[28,45-47]. In order to establish and know the extent of homologous adaptation in the studies, changes in MIC were determined. Capita *et al*.[29] have previously reported a change in MIC following the adaptation of *E. coli* to various antimicrobials such as sodium nitrite and sodium hypochlorite. In that study, the MIC after adaptation was observed to be respectively 2.53 and 1.69 times higher than what it was before adaptation[29]. Similarly, Braoudaki and Hilton[24] reported the stability of adapted *Salmonella* enterica to benzalkonium chloride after sub-culturing in antimicrobial-free broth. An increase in MIC was observed for the adapted cells as compared to the non-adapted cells. Alonso-Calleja *et al*.[30] also reported a higher MIC for adapted *E. coli* cells that were exposed to different biocides after re-culturing in a TSB devoid of biocide. Similarly, the results of the current study agree with other studies on changes in

MIC following adaptation. Furthermore, the rugose variant in *V. cholerae* has been observed to be chlorine tolerant[32]. According to Yildiz and Schoolnik[48] the smooth variant of *V. cholerae* O1 E1 Tor was completely inactivated upon exposure to 3 ppm NaOCl for 5 min. However, under the same experimental conditions, the rugose variant survived and about 5 log CFU $mL^{-1}$ cells were recovered. Another report consistent with these observations was the findings of Rice *et al.*[49]. It was suggested that the smooth variant is chlorine sensitive and in contrast, the rugose variant was observed to be chlorine resistant. In addition, the smooth variant of *Vibrio* was reported to be inactivated with 0.5 mg $L^{-1}$ free chlorine when exposed for less than 20 sec. Contrarily, the rugose variant was exposed to a higher concentration of free chlorine (2 mg $L^{-1}$), under the same growth conditions and cells were still recovered after 30 mins[32]. The majority of these studies supports the findings of the current study, which demonstrated the ability of chlorine-adapted *Salmonella* cells (rugose and smooth) to withstand the homologous stress of chlorine by surviving higher chlorine concentrations than was previously exposed. In addition, it is important to note that the majority of the studies on the rugose phenotype of *Vibrio* suggested that the formation of exopolysaccharide (EPS) was responsible for the resistance patterns to chlorine.

In the current study, exposure to sublethal concentrations of chlorine and subsequent adaptation was determined to influence the strength of biofilms formed by adapted cells as compared to non-adapted control *S. typhimurium* cells. Biofilm formation by microorganisms such as *E. coli*, *Listeria monocytogenes*, *Salmonella enterica*, *S. typhimurium* ATCC14028, DT104 and *V. cholerae* have been well documented[10,15,22,29,48,50-52]. In *V. cholerae*, it was reported that the smooth variant has poor attachment as compared to the rugose variant[48]. The *S. typhimurium* has been reported to have the ability to adhere to different surfaces including stainless steel and acrylic and stronger biofilms are formed on stainless steel compared to other surfaces tested[53]. Regardless of the attachment surface, the great concern must be afforded to chlorine adapted *S. typhimurium* which are capable of forming strong biofilms. The presence of *Salmonella* in biofilms in a food processing plant may not be easily inactivated through the conventional sanitization process. In a previous study, the biofilms of *Salmonella* species formed on a plastic surface was completely inactivated after exposure to 100 ppm chlorine concentration for 20 min[17]. However, in the current study, *Salmonella* cells that formed biofilms were previously adapted to a higher concentration of chlorine even up to 550 ppm, which is substantially higher than the 200 ppm allowed by USDA-FSIS for use during sanitation. Furthermore, a stronger biofilm formation for the adapted rugose cells observed in this study and other studies on *V. cholerae* suggests that, the formation of exopolysaccharide help with the aggregation of cells and can act as a protective covering for the cells[32,35]. It further assists in preventing the bacterial cells from being inactivated by the sanitizer. Additionally, another study presents a different perspective on the rugose morphotype. In the study, rugosity was explained to be correlated with curli and cellulose. The curli represents adhesion and when it combines with cellulose, it allows the cells to adhere to one another[53-55]. The findings in the current study support the suggestions of Morris *et al.*[32] that the production of exopolysaccharide mainly promotes attachment of bacteria as observed in both the crystal violet assay and the enumeration of strongly attached cells. One of the aims of this study was to determine the ability of chlorine tolerant *Salmonella* cells to form biofilms. The finding from this study suggests that more cells of the chlorine adapted rugose variant of *S. typhimurium* attached to the plastic surface tested compared to the smooth variant (adapted and non-adapted), which is worth pointing out because plastic materials are often used in the processing of poultry carcasses. These findings will provide significant information to poultry processors on the right use of chemicals for the purpose of sanitization.

Over the years, there are concerns pertaining to the possibility of cross-adaptation between a previous adaptation to an antimicrobial and antibiotic resistance[25,26]. The global growing concern for the resistance of foodborne pathogens to antibiotic calls for great attention, especially in nations where infections to the pathogens are rampant. Although, only slight differences were observed in the susceptibility patterns of adapted and control cells to the majority of the antibiotics tested, a minimal reduction in susceptibility observed for antibiotics such as penicillin (AMX), cephalosporin (CTX), aminoglycoside (S and GN), quinolones (NA), tetracycline and fluoroquinolones (CIP) is worth pointing out. This is because some of these are the antibiotics of choice in the treatment of *Salmonella* infections especially in adults[56]. Some studies have reported a frequent occurrence in the cross-resistance patterns for fluoroquinolones, quinolones and aminoglycosides against gram-negative bacteria[25,57]. Contrary to the findings in this study, other studies have observed and reported cross-resistance between some antimicrobial agents and antibiotics in *E. coli*, *P. aeruginosa* and some strains of *Salmonella*[25,29,58,59]. The majority of these studies suggested the presence of a common resistance mechanism between adaptations to an

antimicrobial and antibiotic resistance[25,29,60]. The broad objective of this study was to examine whether *Salmonella* adaptation to chlorine stress would induce cross-adaptation to antibiotics. The current study did not find an interaction between chlorine adaptation and antibiotic resistance. However, the findings of this study suggest that the previously adapted rugose variant of *S. typhimurium* may possess a slight reduction in susceptibility to antibiotics. The rugose variant should be examined further to determine the depth and mechanism of adaptation. This will provide information that would be beneficial to food safety and public health.

## CONCLUSION

In conclusion, *S. typhimurium* was adapted to chlorine after being exposed to sublethal concentrations and consequently was tolerant to higher concentrations even above the MIC. This resulted in the formation of rugose and smooth adapted cells, which are both able to form better biofilms than the non-adapted *Salmonella* cells.

## SIGNIFICANCE STATEMENT

The findings of this study show the ability of *Salmonella typhimurium* to acquire adaptation to common sanitizing agent such as chlorine, thus transforming to the rugose variant. The rugose variant, which is more virulent, is capable of strong attachment and subsequently form biofilms on a plastic surface significantly pose a potential threat to food safety. In addition, sublethal exposure of foodborne pathogens to antimicrobials could occur when disinfecting chicken carcasses or sanitizing the processing equipment. This may result in the reduced efficacy of such antimicrobial and reduce the susceptibility of the foodborne pathogen to antibiotics as well. Therefore, the findings of this study suggest that emphasis should be laid on proper cleaning and sanitation. A routine check of sanitizer efficacy and the correct application of sanitizing agent is also recommended.

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