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Salmonella typhimurium and Staphylococcus epidermidis biofilms resistance to Chlorinated water on plastic surface

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Abstract: *Salmonella typhimurium* and *Staphylococcus epidermidis* biofilm persistence was investigated on the plastic surface with different concentrations of chlorinated water. The quantification of bacteria biofilm persistence was performed with 1/20 diluted trypcase soy broth (1/20-TSB) in plastic microtitre plates. The optical densities were determined in each well. The maximal inhibitory activity (IA) was 88.102 for *Salmonella typhimurium* and 92.522 for *Staphylococcus epidermidis*. Higher IA values were obtained at each concentration of chlorinated water for *Staphylococcus epidermidis* than *Salmonella typhimurium*. Both strains have shown their ability of persistence when they form biofilm and the inefficiency of the common dilution of chlorinated water (50- 200 mg/L) to eliminate them completely. The results show that the flagellated Gram-negative bacterium, *Salmonella typhimurium* persistence to the disinfectant agent was higher than the Gram negative cocci, *Staphylococcus epidermidis* in plastic surface. However the effect of the disinfectant agent was not statistically different between the two strains.
Key words: *Salmonella typhimurium*, *Staphylococcus epidermidis*, biofilms, resistance to Chlorinated water, plastic surface

Introduction

Salmonella typhimurium and *Staphylococcus epidermidis* are important pathogens responsible for nosocomial infections. These bacteria are transmitted by food; hence appropriate hygienic safety is needed. Numerous studies have shown that these bacteria are capable of adhering and forming biofilm on metal, glass or rubber surfaces[1,2,3]. *Salmonella* as *staphylococcus* species have been described as environmental persisters[4,5]. The conventional cleaning and sanitation used to eradicate planktonic bacteria may fail with the same strains in biofilm. Previously published reports have suggested that *Salmonella typhimurium* as *Staphylococcus epidermidis* can remain on surfaces and equipments used for handling and washing raw meat. Conventional cleaning and sanitation become ineffective to eradicate the bacteria from such surfaces[6].

The chlorinated water is commonly used to wash fruits, cru food and disinfect food equipment and substrate surfaces. At present, chlorine at a concentration of 50–200 mg/L is the primary postharvest sanitizing agent in routine use in the fresh produce industry[3,7]. This concentration of chlorine is usually ineffective to

eliminate pathogens from leafy vegetables[8,9]. The presence of bacterial biofilms in medical devices, equipment and food processing plants is indicative of a potential source of external contamination[10]. Chlorine treatment at a concentration of 200 mg/L of inoculated lettuce, for instance, reduced less than 2 log of either Listeria monocytogenes, *E. coli* O157:H7 or *Salmonella* population[11]. Chemical treatments, such as calcium or sodium hypochlorite, hydrogen peroxide, ethanol, and a variety of detergents partially reduced the populations of the pathogens on surfaces when they form biofilm[12]. In the present report, we studied the inhibitory activity of chlorinated water in order to elucidate its antimicrobial effect degree against bacilli Gram-negative and cocci Gram positive microorganisms on plastic surface.

Materials and Methods

Bacterial Strains and culture conditions

Two strains, isolated from infants, were used in this study: *Salmonella typhimurium* and *Staphylococcus epidermidis*. The two strains were isolated in the course of routine specimen testing in CHU Kara and were confirmed in CHU Campus bacteriology laboratories in Togo. Preliminary identification of the isolates was based on the Gram stain, morphological and cultural characteristics, catalase, oxydase, coagulase, thermonuclease reaction and hydrolysis of esculin. Isolates with typical cultural characteristics were further identified by conventional biochemical testing and serologic typing. Serogrouping was performed with polyvalent Salmonella antiserum followed by specific O and H antiserum. Antisera were purchased commercially from "Lab Kit".

The strains were stored at -70°C in trypcase soy broth (TSB; Bio-Mérieux, Charbonnières les Bains, France) containing 12.5% glycerol. For inoculation, all strains were transferred from the stock cultures into TSB and incubated overnight at 30°C. All strains were subsequently subcultured one more time under the same conditions. The grown cultures were used for inoculation into medium poured into the wells of plastic microplates for subsequent quantification of biofilm production.

Medium design and quantification of biofilm formation on plastic surface

The medium used in this study was 1/20 diluted TSB autoclaved 25 min at 116°C. Quantification of biofilm production in plastic microtitre plates was based on the previously described method [13]. The wells of a sterile 96-well flat-bottomed polystyrene microplate (Falcon, Becton–Dickinson Labware, Frankin Lakes, NJ, USA; not prepared by the manufacturer for tissue culture work), were filled with 230 µl of the 1/20-TSB medium. A quantity of 20 µl of overnight bacterial culture was added into each well. Each strain was tested in triplicate and the manipulation was performed in duplicate in micro-plates separately. One of the manipulations was used for microscopy and the second for the inhibitory activity (IA) determination. The plates were incubated aerobically for 24 h at 30°C. The content of the plates was then poured off and the wells washed three times with 350 µl of sterile distilled water to remove non-adherent bacteria to the polystyrene surface.

Fluorescent Microscopy

The remaining attached bacteria were fixed with 300 µl of methanol per well, and after 15 min micro-plates were emptied and air dried. The micro-plates were stained with 300 µl per well of Crystal violet used for Gram staining (Gram-colour staining set for microscopy; Merck) for 5 min. Excess stain was rinsed off by placing the micro-plate under running tap water.

Strain persistence and Inhibitory Activity of chlorinated water

After the micro-plates washed were air dried, the dye bound to the adherent cells was used to study the inhibitory activity of chlorinated water.

Sensitivity of Salmonella typhimurium and Staphylococcus epidermidis was expressed as the inhibitory activity, which was determined by the micro-dilution assay previously described by Naghmouchi K *et al*. (2010) [14] using sterile flat-bottom 96-well polystyrene microplates (Falcon, Becton–Dickinson Labware, Frankin Lakes, NJ, USA). The Microplates were loaded with 300µl of 1.5 serial dilutions of disinfectant agent, sodium hypochlorite (NaClO) starting at 1500 mg/L (or 3 times the concentration of commercial solution). After 10 minutes, the content of the plates was poured off and the wells washed with 350 µl of sterile distilled water to remove the inactivated bacteria to the polystyrene surface by the disinfectant. Then, 300 µl of phosphate buffer (PB) were distributed in each well and plates were vortexed for 10 minutes to remove the remained attached bacteria.

Optical densities were read at 600 nm using a Multidetection microplate reader (Technicon, Bio-Mérieux, France). Controls (wells inoculated with the tested culture without added disinfectant agent) were run on each microplate. The microplate assay was repeated at least three times for each disinfectant/bacterial combination, and the IA was the average of the three independent repetitions.

Based on the OD produced by bacterial films at each concentration of chlorinated water, strains were classified into the following categories: no biofilm persistence, weak, moderate or strong biofilm persistence, as previously described[13]. But the particularity of this study was to evaluate the bacterial persistence; so the OD given by the low concentration of chlorinated water was used to define the cut-off OD. Briefly, the cut-off OD (ODc) was defined as three standard deviations under the mean OD of the high serial dilution. Strains were classified as follows:

OD ≤ ODc : no biofilm persistence, ODc < OD ≤ (2 x ODc) : weak biofilm persistence, (2 x ODc) < OD ≤ (4 x ODc) : moderate biofilm persistence and (4 x ODc) < OD: strong biofilm persistence.

The inhibitory activity (IA) of chlorinated water was calculated as a percentage as follows: IA=100-100[OD600(*x*)/OD600(*i*)], where *x* is the culture containing disinfectant and *i* is the control culture.

Statistical analyses

Statistical analyses were performed using the software JMP (SAS Institute Inc.version 5.0.1a, Cary, NC, USA) and Minitab (release 14.2, Minitab Inc., PA. USA).

Results

Quantification of the strains persistence to the disinfectant agent

The strains persistence to chlorinated water was quantified in microtitre plates at different concentrations starting at 39 mg/L to 1500 mg/L. Corresponded OD600 values discarded were respectively ranged decreasing from 1,015 ± 0,080 to 0,121 ± 0,020 for *Salmonella typhimurium* and from 0,985 ± 0,084 to 0,078 ± 0,012 for *Staphylococcus epidermidis* (Table I). At the concentrations above 296 mg/L for *Staphylococcus epidermidis* and 667 mg/L for *Salmonella typhimurium*, the optical densities were respectively under the ODc values (0,252 and 0,240) meaning that there was no biofilm persistence. At concentrations between 198-296 mg/L and 296-667 mg/L respectively for *Staphylococcus epidermidis* and *Salmonella typhimurium*, ODc < OD ≤ (2 x ODc), there was weak biofilm persistence. At concentrations between 39-198 mg/L and 132-296 mg/L respectively for *Staphylococcus epidermidis* and *Salmonella typhimurium*, (2 x ODc) < OD ≤ (4 x ODc), there was moderate biofilm persistence. Under 39 mg/L and 132 mg/L respectively for *Staphylococcus epidermidis* and *Salmonella typhimurium*, (4 x ODc) < OD, there was strong biofilm persistence.

Table I : *Salmonella typhimurium* and *Staphylococcus epidermidis* biofilm quantification

Chlorinated water concentrations (mg/L)	**Biofilms quantification**	
	Salmonella typhimurium (Mean OD/Standard deviation)	*Staphylococcus epidermidis* (Mean OD/Standard deviation)
-	1,017*	1,043*
39	1,015 ± 0,080	0,985 ± 0,084
59	1,017 ± 0,081	0,985 ± 0,084
88	1,004 ± 0,070	0,968 ± 0,075
132	0,952 ± 0,069	0,821 ± 0,055
198	0,782 ± 0,066	0,569 ± 0,062
296	0,587 ± 0,067	0,265 ± 0,029
444	0,429 ± 0,038	0,147 ± 0,019
667	0,235 ± 0,028	0,088 ± 0,010
1000	0,129 ± 0,020	0,077 ± 0,011
1500	0,121 ± 0,020	0,078 ± 0,012

-* (Chlorinated free PBS)

- *Salmonella typhimurium* ODc = 3 x 0,080 x 3 = **0,240**

- *Staphylococcus aureus ODc* = 3 x 0,084 x 3 = **0,252**

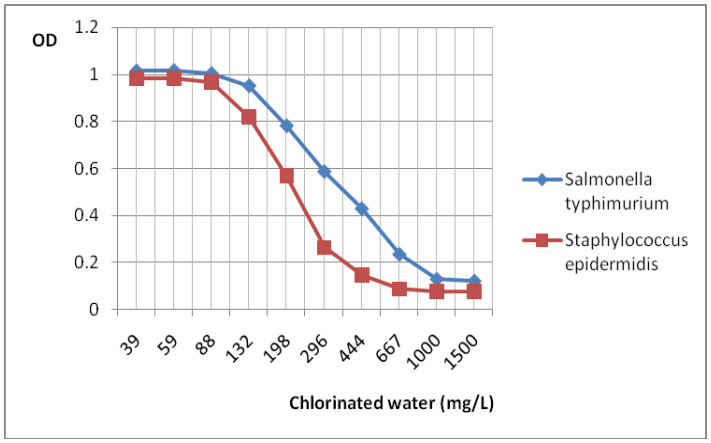

Figure 1: Profile of *Salmonella typhimurium* and *Staphylococcus epidermidis* biofilms persistence at different concentrations of chlorinated water.

On the other hand, the figure 1 shows the OD profile of *Salmonella typhimurium* biofilm above the profile of *Staphylococcus epidermidis*.

Effect of disinfectant agent on biofilm formation

Table II : The Inhibitory Activity of chlorinated water on *Salmonella typhimurium* and *Staphylococcus epidermidis* biofilms

Chlorinated water concentrations (mg/L)	Inhibitory Activity (IA)	
	Salmonella typhimurium	*Staphylococcus epidermidis*
-	0	0
39	0,197	5,561
59	0	5,561
88	1,278	7,191
132	6,391	21,285
198	23,107	45,446
296	42,281	74,592
444	57,817	85,906
667	74,926	91,563
1000	87,316	92,617
1500	88,102	92,522

The maximal IA was 88.102 for *Salmonella typhimurium* and 92.522 for *Staphylococcus epidermidis* (Table II). Higher IA values were obtained at each concentration of chlorinated water for *Staphylococcus epidermidis* than *Salmonella typhimurium*. However the effect of the disinfectant agent was not statistically different between the two strains. The main findings regarding the effect of chlorinated water at different concentrations against aforementioned bacterial biofilms are shown in figure 2. The low inhibitory effects of chlorinated water started significantly at final concentrations of 132-198 mg/L and 198-296 mg/L respectively for *Staphylococcus epidermidis* and *Salmonella typhimurium*. The high IA of chlorinated water was obtained at final concentrations of 444 and 667 mg/L respectively for *Staphylococcus epidermidis* and *Salmonella typhimurium*. For the two strains, significant differences were observed (One-way ANOVA, $p < 0.001$) between the IA of chlorine extreme values.

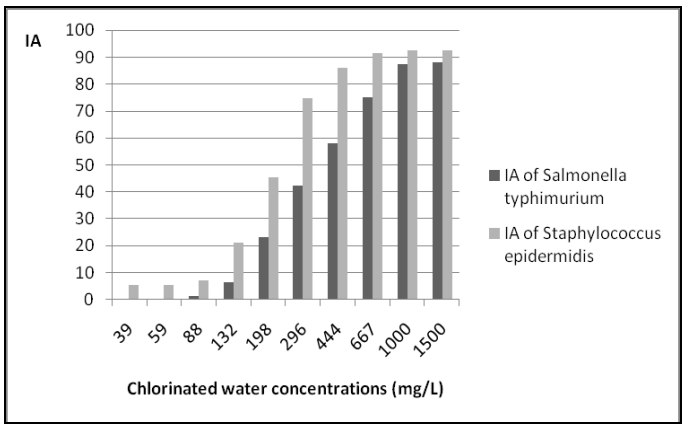

Figure 2: Profile of the Inhibitory Activity of chlorinated water on *Salmonella typhimurium* and *Staphylococcus epidermidis* biofilms

Discussion

Chlorinated water was commonly use for cleaning and sanitation but to our knowledge, no study has been undertaken in developing countries to clarify its effectiveness against resistant bacteria. Now, it is well known that microorganisms in biofilm are less susceptible to antibiotics and disinfectant agents than when they are floating in solution[15,16,17]. This study was to evaluate the persistence of some environmental strains to disinfectant agents.

As a cleaning agent, chlorine is very effective in removing protein residues and, to a lesser extent, carbohydrate material from contaminated surfaces. Cleaning with chlorine removes visible soil and/or food particles from processing equipment and physically reduces the microbial load. The efficacy of chlorine as a bactericidal agent is affected by the pH value of the solution and the amount of organic material present[18,19]. In practice, the efficacy of chlorine in reducing bacterial levels decreases with increasing pH and increasing organic load. During cleaning, organic loads are usually high and alkaline detergents are often used; therefore, chlorine has little antimicrobial activity under these circumstances, and there is little or no hypochlorous acid formed at pH 7.0 or above. The pH values most conducive to the formation of hypochlorous acid are in the pH range 4.0-6.0[19,20,21]; so our study was performed in these conditions.

The reason for the lack of sanitizer effectiveness is still unknown, but was thought to be due to reduction of the oxidizing power of the chlorine by the high organic load of animal carcasses or plants[22,23], or lower accessibility of the target pathogen, that could be achieved by either internalization of the organisms into the tissues, or aggregation and biofilm production on the surfaces. The aim was to determine appropriate concentration of chlorinated water that can be use for an efficient cleaning of plastic surfaces commonly used in food industries and hospitals. After 10 min, attached cells that were able to produce the biofilm matrix were more resistant to the disinfection treatments for concentrations less than 132 mg/L and 198 mg/L respectively for *Staphylococcus epidermidis* and *Salmonella typhimurium* (Table 1). The profiles of biofilms formations decreased significantly at chlorinated water concentrations ranged from 132 mg/L to 444 mg/L for *Staphylococcus epidermidis* and from 198 mg/L to 667 mg/L for *Salmonella typhimurium*. These observations prove that we need almost the commercial disinfectant concentration (500 mg/L) to eliminate completely persistent *Staphylococcus epidermidis* strains. This result also suggested that even non-diluted solution of commercial chlorinated water can't eliminate completely persistent *Salmonella typhimurium.*

Although there is no statistical difference in the inhibitory activity of the disinfectant agent between the two strains, the evaluation of biofilm formation by *Salmonella typhimurium* and *Staphylococcus epidermidis* in this study revealed that these strains possess a high capacity for biofilm formation on plastic surfaces, in terms of resistance. The maximal IA was 88.102 for *Salmonella typhimurium* and 92.522 for *Staphylococcus epidermidis*. These results confirm previous findings, which showed that *Salmonella typhimurium* and *Staphylococcus epidermidis* are able to form biofilm on plastic surfaces[24,25,26,27,28]. However, the significance of this study originates from the fact that we employed essentially a Gram-negative bacillus and Gram positive cocci in the investigation. It has been previously shown that micro-organisms, including *Salmonella typhimurium* and *Staphylococcus epidermidis* strains, adhere in higher numbers to more hydrophobic

materials[29,30,,31]. As adhesion is the first step in complex process of biofilm formation[31]; this could be one possible explanation for the ability of these bacteria to produce biofilm in high numbers on plastic surface.

A biofilm is a structured consortium of bacteria embedded in a self-produced polymer matrix consisting of polysaccharide, protein and DNA[5]. Bacterial biofilms cause chronic infections because they show increased tolerance to antibiotics and disinfectant chemicals as well as resisting phagocytosis and other components of the body's defense system. The persistence of, for example, staphylococcal infections related to foreign bodies is due to biofilm formation. Likewise, chronic *Pseudomonas aeruginosa* lung infection in cystic fibrosis patients is caused by biofilm-growing mucoid strains. Characteristically, gradients of nutrients and oxygen exist from the top to the bottom of biofilms and these gradients are associated with decreased bacterial metabolic activity and increased doubling times of the bacterial cells; it is these more or less dormant cells that are responsible for some of the tolerance to disinfectant agents[32,33,34]. However, obtained results, even not significant statistically, showed that there are differences between the quantities of biofilm produced by the tested *Salmonella typhimurium* and *Staphylococcus epidermidis* strains. The greater biofilm production by *Salmonella typhimurium* than that by *Staphylococcus epidermidis* could be in agreement with the published superiority of Gram negative bacteria to form biofilm on inert surfaces[35,36]. In general, it is assumed that glass and stainless steel are hydrophilic materials while rubber and plastic are hydrophobic materials[30,31]. Consequently, *Salmonella* strains seem more hydrophilic; hence adhere and form more biofilm on plastic surfaces than *Staphylococcus*. On the other hand, *Salmonella* as most Gram negative bacilli possess flagella, pili and curli witch are determinant elements in the exopolysaccharides formation.

Conclusion

This study demonstrated that *Salmonella typhimurium* and *Staphylococcus epidermidis*, food-borne pathogens, readily form biofilm on plastic surfaces, which are nowadays frequently used in food-processing environments. The two strains are commonly involved in nosocomial infections. These bacteria have the potential to spread through fecal waste, potentially contaminating both farm workers and processing plants, food, or the natural environment. An understanding of resistant-bacterial infection to human must take into account the effect of film formed on any substrate, commonly used in food processing environment and in hospitals. This is essential in order to find ways to prevent contamination and to develop strategies of efficient use of chlorinated water for cleaning and sanitation.

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