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ARTICLE

Evaluation of Commercial Disinfectants for Efficacy at Inactivating *Enterobacter sakazakii* (*Cronobacter* spp.) in Water: A Preliminary Study

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Abstract

This study was conducted to evaluate the efficacy of commercial disinfectants at inactivating *Enterobacter sakazakii* (*Cronobacter* spp.) in water. Disinfectant I contained 6.15% sodium hypochlorite, and disinfectant II contained both 2.25% n-alkyl dimethylbenzyl ammonium chloride and 2.25% n-alkyl ethylbenzyl ammonium chloride. Disinfectant I was added to distilled water to obtain a range of residual chloride concentrations at 50 ppm intervals with a maximum of 1-1,000 ppm. Disinfectant II was prepared at concentrations ranging from 1-200 ppm with 5 ppm intervals. Exposure time for all solutions was 10 min. In total, 58 *E. sakazakii* (*Cronobacter* spp.) strains were tested in this study. Nine isolates were obtained from clinical samples, and 49 isolates were obtained from environmental samples. Seven strains (6 clinical and 1 environmental) were able to survive in 100 ppm disinfectant I, and a maximum of 5 ppm of disinfectant II. Fifty one strains (3 clinical and 48 environmental) were not killed in 10 ppm of disinfectant I and 1 ppm of disinfectant II in water. In conclusion, this study demonstrated that clinical *E. sakazakii* (*Cronobacter* spp.) strains displayed 5- to 10-fold higher resistance to disinfectants than environmental *E. sakazakii* (*Cronobacter* spp.) strains. Disinfectant II, containing quaternary ammonium compounds, was shown to be more potent in inactivating *E. sakazakii* (*Cronobacter* spp.) in water used to clean infant formula manufacturing equipment than disinfectant I.

Keywords

Enterobacter sakazakii (*Cronobacter* spp.), disinfectant, sodium hypochlorite, quaternary ammonium compound

Introduction

Enterobacter sakazakii (*Cronobacter* spp.) was the member of the family Enterobacteriaceae [1-6]. Until 1980, *E. sakazakii* (*Cronobacter* spp.) was referred to as yellow pigmented *Enterobacter cloacae* [1,3,4]. Then, *E. sakazakii* (*Cronobacter* spp.) was newly reclassified as a distinct species because it differed from *Enterobacter cloacae* in DNA relatedness, specific yellow pigment production, biochemical reaction, antibiotic susceptibility, and so on [1,3,4,6]. Several outbreaks or sporadic cases of severe neonatal meningitis or necrotizing enteritis in premature infants have been reported due to *E. sakazakii* (*Cronobacter* spp.) [1-6].

In some of these, contaminated dry infant formulas have been identified as the source of *E. sakazakii* (*Cronobacter* spp.) [1-3,5,6]. Since heat treatment such as pasteurization could easily kill organisms, it was presumed that these may be contaminated after

treatment [3,4,7]. Furthermore, it is assumed that *E. sakazakii* (*Cronobacter* spp.) may be present in the environment of processing equipment [1–7].

The vehicle of *E. sakazakii* (*Cronobacter* spp.) was not identified in all cases [1,3–5,7]. Fortunately, dried infant formula was epidemiologically identified as the source of *E. sakazakii* (*Cronobacter* spp.) [1,3–5,7]. As a specific example, *E. sakazakii* (*Cronobacter* spp.) was detected in at least 3 cases of neonatal meningitis and 1 case of necrotizing enterocolitis [1,3–5,7].

To date, very few studies have been published on the resistance of *E. sakazakii* (*Cronobacter* spp.) to commercial disinfectants. According to data, microbial contaminants are generally known as raw materials, factory workers, processing environments, handling equipment, and so on [2,6,8–12]. Also, traditionally, processors used water with or without chemical disinfectants to rinse fresh, minimally processed products [8–10].

In general, sodium hypochlorite (disinfectant I) and quaternary ammonium compounds (QAC, disinfectant II) are widely used as disinfectants in the food industry [8,10,13,14]. As a powerful oxidizing agent, hypochlorite is known to be very active in killing most bacteria, fungi and viruses [13,15]. Because QAC is hydrophilic and negatively charged, it is known that QAC is easily adsorbed to the bacterial surface and then could penetrate the cell wall and destroy the cytoplasmic membrane [13–16]. Oxidative disinfectants are the final barrier to the multi-barrier approach recommended by the US Environmental Protection Agency (EPA) to provide consumers with pathogen-free water [15–16]. Therefore, it is the most commonly used disinfectants for drinking water.

Also, hypochlorite (chlorine) is still used as a disinfectant that is over 100 years old, and most microorganisms are inactivated by hypochlorite [17]. The prevention of introducing and spreading various infectious disease pathogens is the most important step in controlling infectious diseases, and the effective use of disinfectants at this time is a very essential and important measure for controlling the route of infection [18,19]. Also historically, there have been many reports of highly successful uses of disinfectants to control and prevent infectious diseases in humans and animals [17–19].

However, studies on the effectiveness of disinfectants against *E. sakazakii* (*Cronobacter* spp.) are still ongoing. For this reason, there is little information on routine disinfection for protection against *E. sakazakii* (*Cronobacter* spp.). Therefore, there is a need to investigate the effect of representative disinfectants on *E. sakazakii* (*Cronobacter* spp.).

The objective aim of this study was to investigate the impact of QAC-containing detergent (BDD, disinfectant II, Sweden) on susceptibility properties of solution inoculated with *E. sakazakii* (*Cronobacter* spp.) strain isolated from the clinical sample or from the environmental sample to clean the equipment used in preparing infant formula against current-used chlorinated sanitizers (Clorox, disinfectant I, USA).

Materials and Methods

1. Strains

Strains of *E. sakazakii* (*Cronobacter* spp.) were obtained from UGA (Dr. Jeffrey Kornacki, University of Georgia, USA), NRC (Dr. John Marugg, Nestle Research Center,

Switwerland), and FDA (Culture collection of U.S. Food and Drug Administration, USA). A list of all strains with their origin is collected in Table 1. All strains were maintained in glycerol at -80℃. Strains were inoculated individually into trypose soy broth (Difco, Becton Dickinson, USA) to grow overnight (16 to 18 hours) at 37℃, and equal amounts were pooled prior to use as an inoculum. The purity of the culture was confirmed using a biotyping kit (API 20 E: bioMerieux, USA).

2. Disinfectants used in this study

Disinfectant I (Clorox) contained 6.15% sodium hypochlorite, and disinfectant II (BDD) contained both 2.25% n-alkyl dimethylbenzyl ammonium chloride and 2.25% n-alkyl ethylbenzyl ammonium chloride. Disinfectant I was added to distilled water to obtain a total residual chloride of 1-1,000 ppm with 50 ppm intervals, and disinfectant II was prepared at concentrations ranging from 1-200 ppm with 5 ppm intervals for the exposure 10 min, respectively.

3. Preparation of inoculum

Frozen all suspensions of 58 *E. sakazakii* (*Cronobacter* spp.) were thawed and streaked onto tryptic soy agar (Difico, Becton Dickinson, Sparks). The TSA plates were incubated at 37℃ for 24 h before picking colonies to be transferred into 9 mL of tryptic soy broth (TSB). Tubes were incubated at 37℃ for 24 h. A mimimum of two consecutive 24-h transfers were made via loop inoculum (about 10 μL) into 9 mL of TSB before cells were harvested by centrifugation at 3,000×g for 15 min (Centra CL2 centrifuge, International Equipment, USA). The supernatant was decanted and cells were resuspended in 9 mL of sterile 10 mM phosphate buffered saline (pH 7.2). Culture suspensions (100 μL of 10^8 CFU/mL) prepared and were inoculated into 10 mL solution (9 mL of sterile distilled water and 1 mL of disinfectant at different concentration) such that the final population of *E. sakazakii* (*Cronobacter* spp.) was 10^6 CFU/mL.

4. Bacterial enumeration

After following exposure to each concentration of each disinfectant for 10 min, samples were serially (1:10) diluted in sterile 10 mM phosphate buffered saline (PBS, pH 7.2) and the bacteria enumerated by plating 100 μL of the appropriate dilutions onto trypic soy agar (TSA, Becton Dickinson, Sparks) plates. The plates were incubated at 37℃ for up to 2 days. Colonies were counted and results are recorded as log CFU per milliliter.

5. Statistical analysis

Three replicate experiments were performed for each bacterium studied, and all data were analyzed using Statistical Program. Duncan's multiple range test was used to separate means using a level of significance of $p<0.05$.

Results & Disscussion

In this study, planktonic cells with an initial population of ca. 4.8 to 6.3 log CFU/mL were subjected to a sanitizer inactivation test. As shown in Table 1, trends in the inactivation of planktonic cells were exposed to 1 to 1,000 ppm disinfectant I and 1 to 200 ppm disinfectant II. Planktonic cells of *E. sakazakii* (*Cronobacter* spp.) were inactivated rapidly. Seven strains (6 clinical and 1 environmental) can survive in a 100 ppm concentration of disinfectant I, and at the most 5 ppm of disinfectant II. But 51 strains (3 clinical and 48 environmental) were not killed in 10 ppm concentration of disinfectant I and 1 ppm of disinfectant II in water, respectively (Table 1). In clinical *E. sakazakii* (*Cronobacter* spp.) strains, a 0–3.8 log CFU/mL reduction in cell number was noted after exposure to disinfectant I for 10 min at 100 ppm, and 1.7–4.2 log CFU/mL reduction was exposed to disinfectant II for 10 min at 5 ppm (Table 1). In environmental *E. sakazakii* (*Cronobacter* spp.) strains, a 0–1.4 log CFU/mL reduction in cell number was noted after exposure to disinfectant I for 10 min at 10 ppm, and 0–5.8 log CFU/mL reduction was exposed to disinfectant II for 10 min at 1 ppm (Table 1). A greater decrease in the number of planktonic cells was also observed as compared to clinical *E. sakazakii* (*Cronobacter* spp.).

To compare with the concentration of chlorine in Gram-positive, two hundred milligrams per liter of chlorine is the maximum concentration allowed to be used in shell egg wash. Two hundred mg/liter of chlorinated water was also the most effective treatment in reducing the populations of *Listeria* and *Salmonella* (4.6 and 3.03 log CFU per shell egg, respectively for 5-min treatment; data not shown).

The results of other previous studies similar to those of this study was compared and reviewed as follows. Kuo et al. [20] did not recover no viable salmonellae after chlorinated water treatment (200 ppm) for 1 min, and only 50% of the inoculated shell eggs treated with a chlorinated (200 ppm) water wash were negative for *Salmonella* spp. Also, in Gram-negative, Baker et al. [15] reported that *Helicobacter pylori* was significantly more resistant to chlorine than *Escherichia coli*. The difference between the two organisms was more pronounced at higher doses of chlorine [15]. Thus, while exposure to 0.1 ppm of chlorine for 1 min resulted in the 0.3 log reduction in viable *Helicobacter pylori* cells and the 0.9 log reduction in viable *Escherichia coli* cells, exposure to 0.20 ppm chlorine for 1 min was associated with the 1.8 log reduction in viable *Helicobacter pylori* cells and over 4.0 log reduction in viable *Escherichia coli* cells [15]. Shirai et al. [16] studied effects of chlorine, iodine, and QAC disinfectants on several exotic disease viruses. In general, QAC are good bacterial agents, and they are widely used for the disinfection of environmental surfaces [16]. However, precleaning of such surfaces is often necessary, because the effectiveness of QAC is reduced in the presence of soap and organic matter [16]. And Best et al. [21] reported efficacies of selected disinfectants against *Mycobacterium tuberculosis*, and also demonstrated that sodium hypochlorite required an available chlorine concentration of 10,000 ppm before an effective level of reduction could be obtained.

Our results showed that the clinically isolated *E. sakazakii* (*Cronobacter* spp.) was

more resistant to chlorine and QAC than the environmental isolated *E. sakazakii* (*Cronobacter* spp.; $p<0.05$; Table 1). In general, chlorine disinfectants are volatile, so it is already known that using a high concentration is good for the bactericidal effect [10,13,16–18,21]. A similar trend was also observed in this study. In particular, QAC showed a much stronger effect against *E. sakazakii* (*Cronobacter* spp.). Considering the improvement of the bactericidal effect on *E. sakazakii* (*Cronobacter* spp.), QAC is considered to be a very useful disinfectant.

Also, numerous studies conducted over the past 20 years have pointed out that microorganisms may have a variety of genetic and physiological mechanisms to respond to adverse or stressful conditions [22–26]. In particular, microorganisms under stress could exhibit the variety of changes, ranging from minor metabolic changes to more extreme alterations in cellular structure [22–26]. The physiological or structural changes resulting from exposure to moderate or sub-lethal stress allow an organism to survive high stress conditions that could be fatal [23,25,26]. For example, in the food manufacturing facility designed with conditions that inhibit the growth of pathogens and spoilage agents as much as possible, microorganisms could be sublethal when exposed to acid, cold, heat, nutrient depletion, and so on [22–26]. Thus, while these conditions may retard the growth of microorganisms, they may also induce stress-induced cellular changes that allow the organism to persist within these environments [22–26].

In fact, controlling the presence and growth of *E. sakazakii* (*Cronobacter* spp.) species have been very difficult for the food industry, and this difficulty has been attributed in part to the ability of *E. sakazakii* (*Cronobacter* spp.) to grow under high temperature or refrigeration condition [2,8,9,10,23]. Although the vehicle for *E. sakazakii* (*Cronobacter* spp.) has not been identified in all cases, dried infant formula has been epidemiologically identified as the source of *E. sakazakii* (*Cronobacter* spp.) [1,3,5,8,24,26]. Hence, control of *E. sakazakii* (*Cronobacter* spp.) in the food-manufacturing environment has been a challenge despite the fact that this species is sensitive to commonly used chemicals such as QAC and chlorine-based chemical sanitizers. However, studies on the efficacy of fungicides against *E. sakazakii* (*Cronobacter* spp.) cultured under stress conditions are very scarce. Scheepe-Leberkuhne and Wagner [27] revealed that *E. sakazakii* (*Cronobacter* spp.) produced viscous capsular material, and then the organism could form a biofilm on feeding equipment and contact surfaces. Also, Iversen et al. [22] reported the biofilm formation of *E. sakazakii* (*Cronobacter* spp.) in infant formula milk on materials commonly used for infant-feeding equipments and work surfaces. When grown in infant formula milk, *E. sakazakii* (*Cronobacter* spp.) adhered to silicon, latex and polycarbonate to a greater extent than to stainless steel [22]. Namely, these materials were commonly used for infant-feeding equipments and in preparation area [22].

Until now, there are a few reported data relating to the elimination of *E. sakazakii* (*Cronobacter* spp.) biofilms by commercial disinfectants such chlorine, QAC, hydrogen peroxide, ozone etc., as sanitizer. Recently, studies on the control of various microorganisms using several natural substances are being conducted with much interest [28].

Table 1. Effectiveness of disinfectant I and II treatment for inactivating *E. sakazakii* (*Cronobacter* spp.) in water after 10 min exposure (Unit: log 10 CFU/mL)

Strain No. of *E. sakazakii* (*Cronobacter* spp.)	Source	Inoculation volume	Control	Disinfectant I (ppm)					Disinfectant II (ppm)					Type of isolates
			Water	1,000	200	100	50	10	200	50	10	5	1	
2.82	UGA	6.0	5.7	-	-	5.8	5.8	5.6	-	-	-	4.0	5.2	Clinical isolates (9)
2.81	UGA	6.4	6.3	-	-	6.2	6.1	6.1	-	-	-	3.7	5.8	
2.83	UGA	5.6	5.6	-	-	5.0	4.9	5.3	-	-	-	-	5.3	
2.84	UGA	6.2	6.0	-	-	3.9	5.4	6.0	-	-	-	1.8	5.9	
2.41	UGA	5.8	5.8	-	-	2.0	4.5	5.8	-	-	-	2.8	5.7	
2.40	UGA	6.1	5.8	-	-	-	-	3.8	-	-	-	-	1.6	
2.39	UGA	6.1	5.9	-	-	-	-	5.6	-	-	-	-	3.6	
1.91	UGA	6.0	6.0	-	-	-	-	6.0	-	-	-	-	3.3	
2.80	UGA	6.9	6.0	-	-	2.9	6.0	5.8	-	-	-	4.1	5.9	
FSM 298	NRC	6.2	6.1	-	-	6.1	6.2	6.0	-	-	-	3.1	6.1	Environmental isolates (49)
FSM 261	NRC	6.2	6.1	-	-	-	-	4.2	-	-	-	-	4.5	
FSM 274	NRC	5.0	4.6	-	-	-	-	3.2	-	-	-	-	2.5	
FSM 145	UGA	6.1	5.9	-	-	-	-	5.9	-	-	-	-	4.7	
FSM 294	NRC	6.2	6.1	-	-	-	-	6.0	-	-	-	-	3.1	
FSM 273	NRC	5.7	5.7	-	-	-	-	5.6	-	-	-	-	3.3	
FSM 292	NRC	5.9	5.9	-	-	-	-	5.9	-	-	-	-	3.3	
FSM 306	NRC	4.8	4.8	-	-	-	-	4.9	-	-	-	-	1.9	
FSM 265	NRC	5.6	5.9	-	-	-	-	5.8	-	-	-	-	4.2	
FSM 275	NRC	5.8	5.8	-	-	-	-	5.7	-	-	-	-	3.2	
5	UGA	6.0	5.9	-	-	-	-	6.0	-	-	-	-	1.0	
3	UGA	5.6	5.8	-	-	-	-	5.7	-	-	-	-	-	
2	UGA	5.8	6.0	-	-	-	-	5.9	-	-	-	-	2.9	
1	UGA	5.9	6.1	-	-	-	-	5.9	-	-	-	-	6.0	
7	UGA	5.8	6.2	-	-	-	-	6.0	-	-	-	-	3.2	
6	UGA	5.2	5.4	-	-	-	-	5.3	-	-	-	-	2.2	
2.44	UGA	5.3	5.3	-	-	-	-	5.2	-	-	-	-	1.4	
2.43	UGA	5.7	5.7	-	-	-	-	5.9	-	-	-	-	3.8	
FSM 262	NRC	5.9	6.0	-	-	-	-	5.9	-	-	-	-	3.2	
FSM 270	NRC	6.0	5.9	-	-	-	-	5.9	-	-	-	-	3.7	
FSM 271	NRC	5.9	6.0	-	-	-	-	6.0	-	-	-	-	4.1	
FSM 272	NRC	5.8	5.8	-	-	-	-	5.8	-	-	-	-	4.0	
FSM 287	NRC	5.8	5.6	-	-	-	-	5.6	-	-	-	-	3.7	
FSM 290	NRC	6.1	6.1	-	-	-	-	6.0	-	-	-	-	3.7	
FSM 302	NRC	5.7	5.6	-	-	-	-	5.7	-	-	-	-	5.3	
FSM 295	NRC	6.6	5.7	-	-	-	-	5.7	-	-	-	-	4.9	
FSM 293	NRC	5.8	5.5	-	-	-	-	5.6	-	-	-	-	3.3	
FSM 297	NRC	6.1	5.9	-	-	-	-	5.9	-	-	-	-	3.9	
FSM 299	NRC	6.2	6.2	-	-	-	-	6.1	-	-	-	-	3.4	
FSM 303	NRC	6.0	5.8	-	-	-	-	5.8	-	-	-	-	3.2	
FSM 318	NRC	6.1	6.0	-	-	-	-	6.0	-	-	-	-	3.1	
FSM 321	NRC	5.9	5.9	-	-	-	-	5.8	-	-	-	-	5.5	
FSM 323	NRC	6.0	5.8	-	-	-	-	5.8	-	-	-	-	3.0	
2.42	UGA	6.6	6.2	-	-	-	-	6.2	-	-	-	-	2.7	
2.45	UGA	6.2	6.0	-	-	-	-	5.8	-	-	-	-	1.8	
2.46	UGA	6.0	5.5	-	-	-	-	5.5	-	-	-	-	1.0	
2.47	UGA	6.7	6.2	-	-	-	-	6.3	-	-	-	-	2.1	
2.68	UGA	6.7	6.3	-	-	-	-	5.7	-	-	-	-	2.9	
2.69	UGA	6.2	6.1	-	-	-	-	6.0	-	-	-	-	3.2	

Table 1. Continued

Strain No. of *E. sakazakii* (*Cronobacter* spp.)	Source	Inoculation volume	Control	Disinfectant I (ppm)					Disinfectant II (ppm)					Type of isolates
			Water	1,000	200	100	50	10	200	50	10	5	1	
2.70	UGA	6.3	5.6	-	-	-	-	5.9	-	-	-	-	3.8	
2.71	UGA	6.8	6.3	-	-	-	-	6.4	-	-	-	-	3.5	
2.72	UGA	6.4	5.8	-	-	-	-	5.7	-	-	-	-	2.5	
2.73	UGA	6.3	6.0	-	-	-	-	6.2	-	-	-	-	5.8	
2.74	UGA	6.4	6.1	-	-	-	-	6.1	-	-	-	-	2.4	Environmental
2.75	UGA	4.5	4.0	-	-	-	-	4.1	-	-	-	-	-	isolates (49)
2.76	UGA	6.2	5.6	-	-	-	-	5.7	-	-	-	-	4.4	
2.77	UGA	5.9	5.6	-	-	-	-	5.7	-	-	-	-	3.1	
2.78	UGA	5.9	5.8	-	-	-	-	5.7	-	-	-	-	2.5	
2.79	UGA	6.2	5.9	-	-	-	-	5.8	-	-	-	-	2.6	

Disinfectant I contained 6.15% sodium hypochlorite.
Disinfectant II contained both 2.25% n-alkyl dimethylbenzyl ammonium chloride and 2.25% n-alkyl ethylbenzyl ammonium chloride.
UGA, Dr. Jeffrey Kornacki, University of Georgia, Athens, GA; NRC, Dr. John Marugg, Nestle Research Center, Lausanne, Switwerland.

Therefore, it is considered that various related studies using various natural substances that could inhibit or inactivate *E. sakazakii* (*Cronobacter* spp.) should be further conducted.

Conclusion

The results obtained through this study are analyzed as follows. It was demonstrated that the *E. sakazakii* (*Cronobacter* spp.) strain isolated from the clinical sample was more resistant to the disinfectants used in this study than the *E. sakazakii* (*Cronobacter* spp.) strain isolated from the environmental sample. In other words, the *E. sakazakii* (*Cronobacter* spp.) strain isolated from the clinical sample was shown to be 10-fold resistant to disinfectant I and also 5-fold to disinfectant II, respectively. Disinfectant II, known as a QAC, was shown to be more strong to inactive *E. sakazakii* (*Cronobacter* spp.) in water used to clean infant formula equipments than disinfectant I, because the QAC concentrations used in the present study were 200 times lower than the 200 ppm maximum level permissible on food contact surfaces without rinsing. Further study is strongly required to determine the effectiveness for eliminating *E. sakazakii* (*Cronobacter* spp.) biofilm using by commercial disinfectants, and also study that the lethality of stress-adapted *E. sakazakii* (*Cronobacter* spp.) when the microorganism is cultured under ideal conditions.

Conflict of Interest

The authors declare no potential conflict of interest.

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