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Inactivation of Brazilian wild type and enterotoxigenic *Escherichia coli* by chlorine

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The kinetic inactivation parameters of four wild strains and two enterotoxigenic strains of *Escherichia coli* exposed to commercial calcium hypochlorite were determined. The four wild strains (1A, 3C, 4D and 8H) were isolated from lettuce bought in São Paulo (Brazil), and the two enterotoxigenic strains (TR69 and TR101) were originally isolated from human patients. Decimal reduction time 'D', for 10 mg L^{-1} available chlorine at pH 6.8, varied between 71.4 s for the wild strain 4D and 31.3 s for the toxigenic strain. The 'D' values obtained for wild strain 1A exposed to 5.0 mg L^{-1} available chlorine at pH 6.8 varied between 111.1 s and 41.7 s. The 'D' values obtained for *E. coli* strain TR69 exposed to 10 mg L^{-1} available chlorine varied from 15.2 s at pH 5.4 up to 83.3 s at pH 8.2. The use of the most resistant wild strain of *E. coli* as a biological standard assures maximal effectiveness in controlling water contamination by chlorination.

Keywords: *Escherichia coli*; hypochlorites; chlorine; inactivation kinetics

Introduction

Escherichia coli is commonly involved in intestinal infections of Brazilian children [5,11,16,17] and others around the world [6,18,20,21]. The foods most commonly involved in *E. coli*-linked diarrhea outbreaks are raw vegetables and fruits, meat, fish, poultry, milk, candy, rice, coffee substitutes, and undercooked contaminated hamburgers [2,6,11,18,22].

Chlorine is the most widely used water treatment and industrial plant sanitation disinfectant. It is used extensively in food processing establishments in different forms for sanitizing containers and equipment and for washing fresh fruits and vegetables. It is also used in processing nuts, seafood, poultry and red meat [1,2,13,23,27]. Chlorine is used in various forms. However, confusion exists on the proper dosage and what conditions are best to use in produce-processing operations. Chlorine concentration is the main variable to control [7,15].

Variability in the sensitivity of *E. coli* strains to chlorine is important in: (i) designing sanitary measures for destruction of *E. coli* and (ii) predicting the efficacy of existing sanitary systems in preventing *E. coli* infection.

The objective of this research was to characterize the ability of chlorine to inactivate four strains of *E. coli* (1A, 3C, 4D and 8H) isolated from heads of lettuce bought in São Paulo, Brazil, and two enterotoxigenic strains of *E. coli* (TR69 and TR101) isolated from humans.

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Materials and methods

Bacterial strains and maintenance

The wild type *E. coli* strains (designated 1A, 3C, 4D and 8H) were isolated from lettuce (*Lactuca sativa*) samples gathered in restaurants and flea markets in São Paulo, Brazil. The enterotoxigenic *E. coli* strains (designated TR69 and TR101) isolated from humans were obtained from the Department of Food and Nutrition of the University of São Paulo. The numbering, isolation and identification of the *E. coli* strains were done according to American Public Health Association [3] recommendations. The enterotoxigenic *E. coli* strains produced toxins.

Confirmation of colonies as *E. coli* before and after the experiments was based on the results of four biochemical tests: indole production, methyl red, Voges-Proskauer, and use of sodium citrate as sole carbon source, known collectively as the IMViC tests. Production of urease and of hydrogen sulfide was also used. *E. coli* strains were indole- and methyl red-positive and Voges-Proskauer, sodium citrate, urease and hydrogen sulfide negative.

Cultures were kept lyophilized and were also maintained on Tryptic Soy Agar (TSA, pH 7.3, Difco, Detroit, MI, USA). Every 15 days the cultures were streaked onto fresh TSA, incubated at 33°C for 24 h and stored at 4°C. *E. coli* cultures were activated for 24 h at 33°C on Plate Count Agar (PCA, pH 7.0, Difco) before being used in experiments.

Preparation of phosphate-buffered solutions

Sorensen phosphate-buffered solutions were prepared daily with appropriate portions of stock solution A (0.0667 M, Na_2HPO_4) and solution B (0.0667 M, KH_2PO_4) to achieve pH values of 5.4 (3.0 ml A: 97.0 ml B); 6.0 (12.0 ml A: 88.0 ml B); 6.2 (18.5 ml A: 81.5 ml B); 6.5 (31.8 ml A: 68.2 ml B); 6.8 (50.0 ml A: 50.0 ml B); 7.0 (61.1 ml A: 38.9 ml B); and 8.2 (97.0 ml A: 3.0 ml B). The buffer sol-

utions were prepared from double-distilled chlorine demand-free water. They were sterilized by autoclaving them which did not change their pH.

Sodium thiosulfate solution

Sodium thiosulfate (0.1 N) neutralizer stock solution was prepared by dissolving 26 g of $Na_2S_2O_3 \cdot 5H_2O$ and 0.1 g of sodium carbonate in 1-L of freshly-boiled distilled water. This solution was transferred into a 1-L amber bottle, stored at room temperature for about 24 h, and standardized with 0.01 N potassium dichromate [3].

Preparation of chlorinated solutions

Stock chlorine solutions were prepared from a commercial calcium hypochlorite screened powder, and had the following average composition: calcium hypochlorite (10% w/w), sodium chloride (80% w/w), and sodium tripolyphosphate (10% w/w). Stock chlorine solution was prepared by dissolving 30 g of commercial calcium hypochlorite powder in 100 ml of double-distilled water, previously warmed to 40°C. The solution was filter-sterilized using a 0.22-μm pore size filter (Millipore Corp, Bedford, MA, USA), transferred into a 1-L amber bottle and stored at 4°C for a maximum of 30 days. Chlorine solutions were prepared with chlorine demand-free, deionized glass-distilled water and zero chlorine-demand glassware [26].

Chlorinated solutions were prepared by transferring an appropriate amount of stock solution to an amber 1000-ml volumetric flask and bringing the solution to volume with sterile pH-adjusted Sorensen phosphate-buffered solution. The final chlorine concentration and pH value were again determined each time the experiment was performed.

The concentration of total available chlorine in all solutions was determined by the iodometric method [3], which uses thiosulfate titration to detect available chlorine in commercial preparations of calcium hypochlorite solutions. Available chlorine concentrations of 0, 5, 7, 10, and 15 mg ml^{-1} in phosphate-buffered solutions at pH 6.8, and 10 mg ml^{-1} in phosphate-buffered solutions at pH values of 5.4, 6.0, 6.2, 6.5, 6.8, and 8.2, were used against the *E. coli* strains.

Experimental procedure

A 24-h culture of each strain, grown on PCA in four test tubes, was resuspended in 12 ml buffered solution (pH 7.0) and transferred to the surface of 150 ml PCA in a Roux bottle. The culture was incubated for 48 h at 33°C and cells were resuspended in 30 ml of buffered solution at pH 7.0. The suspension was filtered through sterile gauze into an Erlenmeyer flask containing a stir bar and glass beads.

The contents of the flask were diluted to 100 ml using pH 7.0 buffer. The resulting cell suspension was mixed by a magnetic stirrer for 15 min at a temperature of 4°C, and was used in all survival tests. One milliliter of this suspension diluted with 99 ml of chlorine solution gave approximately 10^8 cells ml^{-1}.

Effect of sodium thiosulfate 0.1 N neutralizer on *E. coli* strains

Any adverse effect of the neutralizing solution on *E. coli* strains was also verified. One milliliter of the test suspension of *E. coli* 1A strain was added to a 250-ml Erlenmeyer flask containing either: (a) 99 ml of water, (b) 97 ml of water and 2 ml of neutralizer, (c) 97 ml of concentrated 20 mg L^{-1} chlorine solution and 2 ml of neutralizer, or (d) 99 ml concentrated 20 mg L^{-1} chlorine solution. Viable populations of *E. coli* 1A strain were determined after 15 min.

Effect of different chlorine environments on *E. coli* strains

The procedure used was a modification of the flask method described by Stumbo [25] for thermic resistance tests of microorganisms. A 1000-ml triple-necked flask was used for this purpose. A thermocouple connected to a temperature register was inserted in one of the lateral openings. The central opening was used for the insertion of the shaft of a mechanical agitator. The third opening was used for the insertion of the inoculum and withdrawal of samples.

The flask, containing 500 ml of freshly chlorinated solution, was placed in a thermostatically-controlled water bath at a temperature of 30°C, so that the surface of the reagent solution remained below the level of the warming bath. The contents of the flask were mixed, until a temperature of 30°C was reached.

Five milliliters of the *E. coli* test strain suspension were inserted in the flask, the contents of the flask were mixed and allowed to react for 10.0 min. Measurements of total available chlorine were made before and immediately after the chemical treatment. The final pH of each solution was also determined. There was no variation detected in either total available chlorine (10.00 ± 0.03), or pH (standard deviation = ± 0.01).

At 30-s time intervals, 5 ml of the sample mixture were withdrawn, transferred to a test tube containing 0.2 ml of neutralizing solution and 4.8 ml of sterile water, and immediately cooled in an ice water bath. Test suspension concentrations of viable *E. coli* strain cells were determined before and after each chemical treatment.

E. coli strain suspension viability

Each sample was homogenized by tube vibrator. The density of the surviving cells was determined by preparing pour plates on TSA and incubating them 24–48 h at 33°C. The serial decimal dilutions for each cell suspension were prepared with 0.1% (g L^{-1}) peptone salt dilution fluid [3]. The mean number of surviving cells ml^{-1} of suspension was determined from the analyses of at least ten plate counts. Analysis of variance was used to determine statistical differences ($P <= 0.05$) [9].

Survivor curves

Survivor curves were generated by fitting the observed data to a linear function that allows for the presence of a prior exponential decline in population density for an initial period of uncontrolled experimental *E. coli* inactivation [8,26].

The survival curve equation generated by fitting the data to the linear function of an exponential decline in population density, is given by:

$$Y = Y_0 - b * (T - t_r)$$

where:

Y = log count of bacteria at time t. Log(CFU ml^{-1});
Y_0 = log count of bacteria at time $t = 0$. Log(CFU ml^{-1});
b = slope of the survivor curve. [Log(CFU ml^{-1}) s^{-1};
t = time (s);
t_r = duration of uncontrolled microbial activity of hypochlorite buffered over a pH range, prior to the experimental period of *E. coli* inactivation.

Results and discussion

The effect of thiosulfate on E. coli cells

The antimicrobial effect of available chlorine was inhibited by mixing with sodium thiosulfate neutralizer. Populations of viable *E. coli* cells exposed to 0.1 N sodium thiosulfate for up to 15 min did not differ significantly from cells in water (data not shown). The sodium thiosulfate was therefore assumed to have no effects on *E. coli* cell population viability.

Survivor curve D-values

The population density of the *E. coli* strains declined exponentially from the initial period t_r of uncontrolled microbial inactivation (Figure 1). An initial lag phase of the survivor curves [4,10] was not observed in any of the reaction environments studied.

The death of the bacterial cell depends on the penetration of chlorine into the interior of the cell. Treatments that increase the cell's permeability to chlorine [4,6,12,19] should increase the death rate and reduce the time required to inactivate the target microorganism. The commercial calcium hypochlorite used in this study contained tensoactive substances, which may have contributed to the reduction of the cellular surface tension, and thus facilitated a more rapid contact between bacteria and available chlorine.

The linear regression analyses were generated for *E. coli* strains exposed to chlorine solutions (Table 1) from the number of mean survivor cells (log CFU ml^{-1}). Decimal reduction times (D-values) were estimated from the negative reciprocal of the slope (–b) of the linear regression survival curve equations [25]. The slope standard error [SE(b)] and correlation coefficient (r^2) were also calculated [9]. The estimated value of the D-value standard error [SE(D)] was obtained from the square root of the variance [V(D)], which was calculated by the equation:

$$V(D) = \left[\frac{1}{-b^2}\right]^2 [SE(b)]^2$$

The upper [D_{mean} + SE(D)] and lower [D_{mean} – SE(D)] D-values were also calculated.

Fits of the linear models were visually acceptable and the significance of 95% D-value confidence intervals confirmed this [$t_{calc} > t_{critical}$ for ($\alpha/2 < 0.025$)] [9].

The behavior of the *E. coli* cells during treatment with chlorine has been shown to be more repeatable as long as the time period considered is greater than 30-s exposure and not longer than 3 min. We observed that 'tailing off' kinetics were not due to the presence of demand substances. This view is also supported by the research of others [14,24,26].

D-values for 10 mg L^{-1} available chlorine at pH 6.8, varied between 71.4 s for the wild strain 4D and 31.3 s for the toxigenic TR101, among all strains studied (Table 1). The D-value of the wild-type strain 1A (50.0 s) most closely approximated the D-value for the most chlorine-resistant toxigenic strain, TR69 (55.6 s).

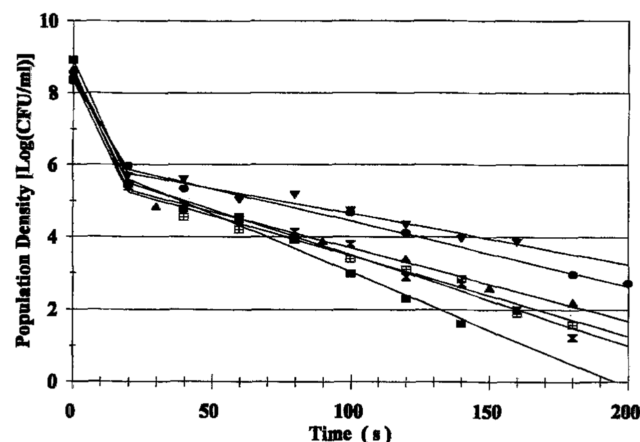

Figure 1 Population density [Log(CFU ml^{-1})] calculated by fitting the observed data to the equations of survival curves for *E. coli* strains 1A ▲, 3c ⊞, 4D ▼, 8H ⧗, TR101 ■, and TR69 ● exposed to 10 mg L^{-1} of available chlorine in pH 6.8 phosphate-buffered solutions.

The effect of total available chlorine concentrations

The mean D-value was reduced by about half when the available chlorine concentration was doubled from 5.0 mg L^{-1} to 10 mg L^{-1}, and from 7.0 mg L^{-1} to 15.0 mg L^{-1} for the *E. coli* strain 1A at pH 6.8 (Table 1). Bacterial cells readily combine with free chlorine and therefore may exert a significant chlorine demand. Ridgway and Olson [24] noted that under the experimental conditions studied, the bacterial contribution to the total chlorine demand was insignificant and our results confirmed this.

The inactivation experiments with chlorine were performed at 30°C, which is the typical ambient air temperature found in the food preparation areas of industrial restaurants in São Paulo city which are not supplied by forced air. That temperature did not have a considerable effect on the efficacy of the chlorine; the effect of temperature is probably overcome by the greater germicidal potency of the chlorine [7,8,12,27].

The effect of pH on chlorine activity

The pH of the test solution is one of the most important factors affecting chlorine sanitizer activity. Formation of hypochlorous acid (HOCl) and of hypochlorine ion (OCl^-), both responsible for antimicrobial chlorine activity, is a function of the pH value solution [10,14,26].

The bactericidal effectiveness of total available chlorine increased as pH decreased (Table 1). When the hydrogen ion concentration increases, the ionization of the acid decreases, increasing the sanitizer action, since the hypochlorous acid form has greater antimicrobial activity [8,10]. The hypochlorous acid form also has an elevated ability to diffuse through the cellular membrane compared to the hypochlorine ion form [4,14,24,26,27].

Decreasing pH values from 8.2 to 5.4 reduced the mean

Table 1 Linear Regression Analysis. Decimal reduction times (mean, lower, upper D-values) determined for *Escherichia coli* strains, exposed to chlorine concentrations (mg L^{-1}) at various pH levels in buffered solutions

pH	mg L^{-1}	Strain	−b	SE(b)	r^2	D-value(s)			t_{calc}
						mean	lower	upper	
5.4	10	TR69	0.066	0.0046	0.986	15.2	13.3	17.1	14.35
6.0	10	TR69	0.043	0.0066	0.955	23.3	19.7	26.9	6.52
6.2	10	TR69	0.040	0.0041	0.970	25.0	22.4	27.6	9.76
6.5	10	TR69	0.026	0.0008	0.999	38.5	37.4	39.7	33.33
8.2	10	TR69	0.012	0.0008	0.981	83.3	77.6	89.1	14.50
6.8	5	1A	0.009	0.0010	0.942	111.1	98.8	123.4	9.00
6.8	7	1A	0.011	0.0009	0.970	90.9	78.7	103.1	12.22
6.8	10	1A	0.020	0.0005	0.994	49.9	48.6	51.1	40.00
6.8	15	1A	0.024	0.0011	0.989	41.7	39.8	43.6	21.82
6.8	10	TR101	0.032	0.0018	0.984	31.3	29.5	33.1	17.78
6.8	10	8H	0.025	0.0012	0.984	40.0	38.1	41.9	20.80
6.8	10	3C	0.022	0.0013	0.977	45.5	42.8	48.1	16.90
6.8	10	TR69	0.018	0.0007	0.992	55.6	53.6	53.4	25.70
6.8	10	4D	0.014	0.0005	0.988	71.4	68.9	74.0	28.00

(−b) = slope.
SE(b) = slope standard error.
D-value = Decimal reduction time (s) = (−1/b).
$[D_{mean} - SE(D)]$ = lower D-value.
$[D_{mean} + SE(D)]$ = upper D-value.
SE(D) = standard error of D-value = $\{(-1/b^2)^2 \times [SE(b)]^2\}^{1/2}$.
t_{calc} = calculated student's t = [b/SE(b)].
$t_{critical}$ = [(0.05/2) (n − 2)].
$t_{critical}$ ranges from 2.306 (n = 10) to 3.182 (n = 5).

D-values of the toxigenic *E. coli* strain TR69 exposed to 10 mg L^{-1} available chlorine, from 83.3 s to 14.2 s (Table 1), confirming previous studies [6,14,26].

The efficacy of chlorine-sanitizer

Escherichia coli is more resistant to chlorine than many other vegetative bacteria, including other pathogens. It can be used as a test organism in food industry sanitizing programs and to test the efficiency of newly developed sanitizers. Few investigations have been made to verify the high efficiency of commercial sanitizers against wild strains of *E. coli* [15,26]. Studies concerning disinfection by inorganic chlorine and chloramines, using strains of *E. coli* have been published by Ridgway and Olson [24], and Ward *et al* [26]. Our results support and reinforce their observations.

Raw fruits and vegetables are a likely cause of illness in Brazilian food service establishments. A good cleaning and sanitizing program should be carefully followed by these establishments to reduce the incidence of food poisoning. The results of this research suggest considerations which should be noted in the development of food service sanitation programs for the Brazilian food service industry.

Conclusions

Decimal reduction time 'D', for 10 mg L^{-1} available chlorine concentration at pH 6.8, varied between 71.4 s for the wild strain 4D and 31.3 s for the toxigenic TR101, among all strains studied, showing that a wild strain should be used as a standard of the effectiveness of a treatment. The use of the most chlorine-resistant strain (*E. coli* 4D) as biological standard assures the effectiveness of the controlling toxigenic strain contamination by washing fruits and vegetables in chlorinated water.

The results indicate that wild-type *E. coli* inactivation is generally similar to pathogenic strain inactivation. Wild-type strains would out-number pathogens in most instances of fecal contamination, so monitoring for wild-type *E. coli* would be sufficient for determining the post-sanitization presence of ETEC.

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