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RESEARCH NOTE

Effects of chlorine on cells of *Vibrio cholerae*

O. V. Sousa[1], R. H. S. F. Vieira[1], T. R. Patel[2*], E. Hofer[1] and V. P. Mesquita[1]

Since the sixth pandemic outbreak of cholera, chlorine had been used as the first line of defense. The objective of this investigation was to determine the bactericidal concentration of chlorine against Vibrio cholerae *in artificially-contaminated samples of shrimps and water. Cells of* V. cholerae *were exposed to varying concentrations* (*5–10 ppm*) *of chlorine in solution for 5 min. Artificially-contaminated shrimp* (Xiphoenaeus kroyer) *samples were also dipped for 5 min in chlorine solutions (10 ppm). The results strongly indicate that 8 ppm chlorine was effective in killing viable cells from pure cultures. Isolates of this organism were recovered from carapace, head and tail parts of the crustaceans.* V. cholerae *strains re-isolated from carapace/head samples seemed to be more resistant than those from tail meat when exposed to 10 ppm chlorine solution for 5 min.*

Introduction

Vibrio cholerae, are prokaryotic members of the marine environment and are causative agents of cholera in humans. Numerous human infections have been documented worldwide. (Albert et al. 1993). A variety of foods, including raw or undercooked shell fish and other sea foods have been implicated in outbreaks of cholera (Organizacao Panamericano de Saude , OPS 1993, Nair et al. 1993). Between 1991 and 1993 a major outbreak in South American countries resulted in more than 6000 deaths in Peru, Ecuador, and Columbia (Popovic et al. 1993). As a consequence many importing countries have been concerned about contaminated seafood imported from countries where *V. cholerae* are detected. A variety of foods, including raw or incompletely-cooked shellfish and other sea foods have been implicated in outbreaks of cholera (Tauxe et al. 1992). Chlorine is widely used as domestic disinfectant and sanitizing agent in food processing industry(WHO 1984). During the last endemic of cholera in Brazil, the Federal Ministry of Agriculture recommended that all seafood processing plants increase their treatments with chlorine to concentrations ranging from 5 to 10 ppm. This was aimed at preventing risk against *V. cholerae*. This investigation focused on the efficiency of chlorine against *V. cholerae* cells in suspension as well as those attached to artificially contaminated sea foods.

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[1]*Marine Science Laboratory, Federal University of Ceara, Fortaleza, CE., Brazil*
[2]*Department of Biology, Memorial University of Newfoundland, St. John's, Nf., A1B3X9 Canada*

Materials and Methods

Organisms

A pure culture of *V. cholerae* 01 was obtained from the culture collection of Oswaldo Cruz Institute and maintained on tryptic soy agar

*Corresponding author. Fax: 709-737-3018.
E-mail: tpatel@morgan.uc.mun.ca

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(TSA) containing 1% sodium chloride. Broth cultures were initiated by an overnight growth at 35°C in tryptic soy broth (TSB) containing 1% sodium chloride. For all tests, freshly-grown (18 h) cultures were used.

Preparation of working solutions

A stock with 2% chlorine was diluted to the required concentrations using chlorine-free water. The content of total available chlorine (TAC) was determined using an iodometric method (APHA 1989).

Shrimp samples

Shrimp samples used in the present investigation were procured from Mucuripe Sea food Market, Fortaleza, CE, Brazil. The shrimp samples were washed and then immersed in 4% formol solution for 3 min before being used in tests.

Effects of chlorine on V. cholerae *cells*

Cell suspensions (1 ml) of freshly grown (18–20 h) cells of *V. cholerae* was added to 100 ml of sodium chloride solution containing 5–10 ppm of chlorine. Control tubes contained sterile water free of chlorine. After exposing the cells for 5 min they were serially diluted and plated in triplicates in tryptone-glucose-extract (TGEA, Difco, Detroit, MI, US) using a pour plate method. The plates were incubated at 35°C for 48 h before colony counts were recorded.

Bactericidal effects of chlorine solutions

Approximately 1 kg of raw shrimps were suspended in 1000 ml of cell suspension of *V. cholerae* in a presterilized beaker. After 5 min the shrimp samples were removed and excess liquid was allowed to drain off. Shrimps were then weighed in 25 g portions. Shrimps (25 g) immersed in 100 ml chlorine-free water were used as a control. After 5 min exposure to chlorine the samples were removed and blended in 225 ml sterile saline solutions. Serial dilutions were prepared and plated in triplicates on TGEA plates using the pour plate method. Colonies of surviving bacteria were counted after incubation of the plated at 35°C for 24–48 h.

The remaining contaminated shrimp samples were stored in a refrigerator at 0–4°C for 6 h. The above experiment was repeated using the stored shrimp samples.

In another test contaminated shrimps were used to separate head/carapace and tail meat portions. These separated parts were immersed in a chlorine solution (10 ppm) for 5 min. Samples were then blended and plated as described above. The surviving bacteria were counted after incubation of plates.

Statistical analysis

Bacterial number in cfu g^{-1} sample were converted into $\log_{10}$ for statistical analysis. An analysis of variance (ANOVA) and Tukey's test. Dunnett's test ('T') was also employed to compare the average number of surviving cells on shrimp head/carapace and tail meat according to the earlier reports (Gomes 1970; Centeno 1990).

Results and Discussion

Table 1 presents the average number of *Vibrio* cells that survived exposure to varying concentrations of chlorine. Chlorine concentrations greater than 7 ppm resulted in 100% reduction in survival (Figure 1). It is evident from the figure that *Vibrio* cells suspended in water are

Table 1. Changes in cell numbers after exposure to varying concentrations of chlorine

Cl_2 conc. (ppm)	$\log_{10}$ cfu ml^{-1} Initial	After treatment	% Reduction
5	7·0	2·43	65
6	7·0	2·08	70
7	7·0	1·25	82
8	7·0	<0	100
9	7·0	<0	100
10	7·0	<0	100

Vibrio cholerae cells were exposed to different concentration of chlorine for 5 min. The surviving bacteria were counted by plating serial dilutions on TGEA plates incubated at 35°C for 24–48 h.

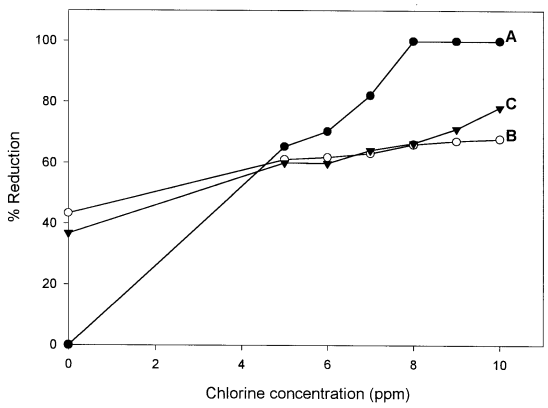

Figure 1. Bactericidal effects of chlorine (5 ppm) on *V. cholerae* cells (A) in a water suspension; (B) those adhering to shrimps; (C) those attached to shrimps stored in cold for 6 h.

Table 2. Reduction in cell numbers of *Vibrio cholerae* contaminated shrimps exposed to chlorine in solution

Cl_2 conc. (ppm)	Log_{10} cfu ml^{-1} Initial	After treatment	% Reduction
0	7·0	3·96	
5	7·0	2·72	61·1
6	7·0	2·67	61·9
7	7·0	2·58	63·1
8	7·0	2·38	66·0
9	7·0	2·30	67·1
10	7·0	2·25	67·9

Artificially contaminated shrimp samples were dipped in varying concentrations of chlorine solutions for 5 min. Surviving bacterial cells were counted by plating on TGEA plates incubated at 35°C for 24–48 h.

much more sensitive to chlorine (5 ppm) than those adhering to shrimp. Cells that resisted chlorine gave rise to 'rugose' colonies, which differed from the typical form exhibited by normal vibrios (small, round, and translucent colonies). The rugose form may have an added advantage in the presence of chlorine which eliminates sensitive forms. This finding becomes of extreme importance when considering the health of general public (Deb et al. 1986)

The survival of *V. cholerae* cells associated with shrimp is presented in Table 2. After 5 min exposure to chlorine, the difference in the total reduction of *V. cholerae* between the lowest (5 ppm) and highest (10 ppm) concentrations did not reach one logarithmic cycle. This observation was reproducible in three repeated experiments. It is possible that this low reduction in the viable cells is associated with the capacity of the bacteria to adhere tightly to chitin in the shrimp exoskeleton. It is known that when in the presence of excess organic matter, chlorine has its bactericidal property reduced (Alcamo 1994). The ability to attach to the carapace of crustaceans, including copepods, is characteristic of the genus *Vibrio* (Pruzzo et al. 1996). The chitinase activity may increase the affinity of the vibrios for animal carapace. This may explain why vibrios are found more frequently in crustaceans when compared to other sea food products (DePaola 1981). Liu and Xie (1994) also found that cells of *V. cholerae 01 El Tor* and non-01 multiplied *in vitro* while adhering to chitin. The chitin provided protection to the micro-organisms subjected to heating, between 56°C and 75°C. for 60 to 160 seconds. Recovery of viable cells of *Vibrio*, attached to chitin, after 10 min exposure to free chlorine, ranged from 3·5 and 15 ppm in concentration.

Other investigators (Uboldi Eiroa and Porto 1996) have emphasized the influence of bacterial adherence to surfaces and their susceptibility to sanitizing agents. Even short time attachment of bacteria to surfaces renders them resistant to sanitizing agents. (Hola et al. 1990). These authors reported that attached cells were about 300 times more resistant to microbicidal agents than non-attached cells. The exact nature of this resistance mechanism is not known.

A chlorine-resistant form of vibrios isolated in the last epidemic of cholera in South America were shown to give 'rugose' colonies (Rice et al. 1993; Morris et al. 1996; Clarke et al. 1994). It was noticed that these rugose survival forms tolerated 30 min exposure to 2 mg L^{-1} chlorine. The recommendation from the Brazilian Federal Ministry of Health (Directive No. 36, January 19, 1990) states that the minimum concentration of free residual chlorine in the municipal water system should be at least 0.2 mg L^{-1}. However, the findings of the present investigation suggests that *Vibrio*

cholerae cells can easily survive under these conditions.

Since chlorination of water is one of the first line of defence against cholera, it is important to understand the survival of the rugose form of bacteria in the presence of chlorine (DePaola et al. 1981). It is evident from the present study that *V. cholerae* cells may be reduced in numbers by chlorine but are not completely eradicated, leaving the potential for contamination and infection.

Shrimp samples contaminated with vibrio cells and stored at 0–4°C for 6 h showed 59·8% and 78·1% reduction in viable cells compared to 36·8% of cells in control samples (Table 3). This is in contrast to results shown in Table 2 where *Vibrio* cells with only a short contact time with the chitinous material did not survive concentrations of chlorine greater than 7 ppm. The data in Table 3 also indicates that storage of *Vibrio* cells at low temperature may have influenced their survival rate.

Lin et al. (1996) reported that cells of *Listeria monocytogenes* contaminating fish blocks required 100 ppm chlorine for a 99% reduction in cooled water. Table 4 reveals that *V. cholerae* cells associated with carapace/head survived better (49·8% reduction) than those contaminating shrimp tail flesh (56·1% reduction). Earlier Nascimento et al. (1998) demonstrated that cells associated with whole shrimps showed significantly higher survival rates than those adhering to shrimps without carapace when subjected to freezing and boiling treatments. The physical protection offered by hollow spaces in the carapace as well as and the chitinous nature of the exoskeleton were two important factors contributing to survival rate.

Table 3. Changes in *V. cholerae* loads of shrimp samples refrigerated for 6 h and treated with varying concentrations of chlorine

Cl_2 conc. (ppm)	Log_{10} cfu ml^{-1} Initial	After treatment	% Reduction
0	7·0	4·42	36·8
5	7·0	2·80	60·0
6	7·0	2·81	59·8
7	7·0	2·51	64·1
8	7·0	2·35	66·4
9	7·0	2·02	71·4
10	7·0	1·53	78·1

Shrimp samples artificially contaminated with *V. cholerae* cells were stored at 0–4°C for 6 h prior to chlorine treatment. After exposure to chlorine solutions for 5 min the surviving bacteria were counted by plating on TGEA plates. Plates were incubated at 35°C for 24–48 h.

Table 4. Differences in the resistance of *V. cholerae* cells attached to various parts of the shrimp

Sample	Log_{10} cfu g^{-1} Initial	After treatment	% Reduction
Carapace/head	7·0	3·51	49·8
Tail flesh	7·0	3·07	56·1
Control	7·0	6·67	4·7

Bacterial cells adhering to different parts were exposed to solutions containing 10 ppm chlorine.

With respect to chemical significance of rugose form of *V. cholerae*, Rice et al. (1993) discovered that these cells were very efficient in their ability to adhere to the erythrocytes as well as cause diarrhoea in the same way as the normal strains. Morris et al. (1996) demonstrated that similarity exists between the rugose forms and smooth (normal) vibrios with respect to clinical symptoms and immunological response to patients.

According to the current recommendations of the Brazilian authorities and supported by WHO (1984), a concentration of 10 ppm chlorine is necessary to eliminate vibrios in water. However, this concentration is insufficient to destroy vibrios adhering to surfaces of shrimp or other crustaceans. More in-depth investigation is needed to determine the chlorine concentration required to destroy the vibrio cells attached to exoskeletons of crustaceans. The rugose strains may contribute to the survival of the organisms under these conditions.

The findings of this study indicates that vibrio cells can resist chlorine concentrations up to 7 ppm. In the presence of chlorine, a variant form develops. Viable cells contaminating shrimp samples were recoverable after 5 min exposure to varying concentrations of bactericidal agent. Comparison of results from Tables 2 and 3, and Fig. 1, suggest that longer contact time of microbial cells with carapace results in greater resistance to chlorine.

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