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Inactivation of *Helicobacter pylori* by Chlorination

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Three strains of *Helicobacter pylori* were studied to determine their resistance to chlorination. The organisms were readily inactivated by free chlorine and should therefore be controlled by disinfection practices normally employed in the treatment of drinking water.

Helicobacter pylori is a gram-negative, microaerophilic, spiral bacterium which infects the human stomach and upper gastrointestinal tract. The organism is an etiological agent of gastritis and has been implicated in the pathogenesis of peptic and duodenal ulcers (13) and the development of gastric carcinomas (5). The mode of transmission of *H. pylori* remains an area open to discussion. Various studies have pointed to the role of poor sanitation in the spread of this organism (16). A report on the presence of *H. pylori* in the feces of children in an area of endemicity (14) is suggestive of a fecal-oral route of transmission. Increased risk factors for infection have been associated with drinking water (10) and the consumption of uncooked vegetables irrigated with untreated sewage (9).

Currently the role of water in the dissemination of this pathogen remains problematic. *H. pylori* is a fastidious organism and consequently has been difficult to isolate from environmental sources. Occurrence studies in the aquatic environment have relied upon molecular methods employing PCR amplification procedures. By using the PCR technique, *H. pylori* has been found to occur in sewage (12) and drinking water (7). Results from seeding studies using PCR coupled with immunomagnetic separation and autoradiographic and cultural methods (4, 11, 17) suggest that the organism may survive in water for an extended period of time. These findings support the possibility that this organism is spread by water. Since chlorine disinfection of drinking water remains one of the primary means of preventing the spread of waterborne disease (2), this study was designed to determine the resistance of *H. pylori* to inactivation by chlorination.

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Three isolates of *H. pylori* were used in this study: two clinical isolates (CP41 and CVD 33, kindly provided by J. G. Morris, Baltimore Veterans Administration Hospital, Baltimore, Md., and M. Shahamat, University of Maryland, College Park) and one isolate (ATCC 43504) from the American Type Culture Collection, Rockville, Md. The organisms were propagated on blood agar plates (tryptic soy agar supplemented with 5% sheep blood) incubated at 35°C for 4 to 5 days under microaerophilic conditions. Organisms were aseptically removed by scraping with a bacteriological loop (11). In order to obtain sufficient numbers of organisms, the scrapings from an entire plate were added to one culture tube containing 9 ml of brain heart infusion broth supplemented with 1% (wt/vol) yeast extract and 20% (vol/vol) sterilized horse serum. Rod-shaped cells comprised approximately 85% of the *H. pylori* inocula. Tubes were incubated microaerophilically for 4 to 5 days at 35°C. Four tubes of each culture were prepared for inoculation for each experiment. Cultures were concentrated and washed three times by centrifugation with the appropriate chlorine demand-free (CDF) 0.05 M potassium dihydrogen phosphate buffer adjusted to the various pH levels examined (1). A recent fecal isolate of *Escherichia coli* was used for a comparative evaluation of the effect of cultural preparation conditions on the presence of particulate matter in the inocula. The *E. coli* culture was grown under the same broth conditions but consisted of an initial small inoculum from an agar slant, as opposed to the scrapings of an entire agar plate.

Inactivation experiments were conducted at 5°C in a recirculating, refrigerated water bath. The reaction vessels consisted of 600 ml of borosilicate glass beakers containing 200 ml of chlorinated CDF buffer. The chlorinated CDF buffer was prepared by the addition of reagent-grade sodium hypochlorite to obtain the desired chlorine concentration. Reaction vessels were continuously mixed (250 rpm) by using an overhead stirring apparatus equipped with sterile stainless steel paddles. All glassware, buffers, and paddles were cooled to refrigeration temperatures prior to use in the experiments. Vessels were inoculated with the various cultures to yield an initial density of between 4.19 and 5.48 $\log_{10}$ CFU/ml. Chlorine levels were adjusted to compensate for any chlorine demand exerted by the inoculum. Initial chlorine levels, prior to the addition of the inocula, averaged 0.54 ± 0.05 mg/liter (mean ± standard deviation). Final free-chlorine residuals averaged 0.50 ± 0.05 mg/liter at the maximum exposure time (80 s). Chlorine concentrations were determined by the *N*,*N*-dimethyl-*p*-phenylenediamine colorimetric method (6). Aliquots were removed from the reaction vessels (test and control beakers) at appropriate exposure times and the disinfectant was neutralized by the addition of 0.5 ml of 10% (wt/vol) sodium thiosulfate. Vessels containing CDF buffer and sodium thiosulfate served as the controls for determining the initial inoculum of the organisms at each pH level. Buffer and thiosulfate controls were also included. Triplicate experiments were conducted for each isolate at each pH level. The disinfectant concentrations (in milligrams per liter) were multiplied by the exposure times (in minutes) as previously described (8); the resulting values are referred to as CT values.

Initial densities and the number of survivors were determined by the spread plate procedure (6). Blood agar plates were inoculated in duplicate at appropriate dilutions for each exposure time. A volume of 0.1 ml was inoculated per plate. Plates were incubated in a microaerophilic environment for 4 days at 35°C. Washed cultures of *H. pylori* and *E. coli* (each containing 10^4 organisms/ml) were examined microscopically with a hemocytometer and calibrated microscope to determine

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TABLE 1. Chlorine inactivation of *H. pylori* in chlorine demand-free chlorinated buffer, 5°C, 0.5 mg of free chlorine per liter

Isolate	pH	Log_{10} CFU/ml				
		Initial inoculum	After exposure time of:			
			10 s	20 s	40 s	80 s
43504	6	4.91 ± 0.02	2.64 ± 0.05	2.36 ± 0.04	1.30 ± 0.10	<0.20
	7	4.63 ± 0.03	3.90 ± 0.05	3.60 ± 0.04	1.78 ± 0.04	<0.20
	8	4.93 ± 0.02	3.89 ± 0.03	3.54 ± 0.03	2.18 ± 0.02	<0.20
CVD33	6	4.74 ± 0.03	2.65 ± 0.03	2.43 ± 0.04	1.95 ± 0.01	<0.20
	7	4.74 ± 0.03	2.78 ± 0.02	2.56 ± 0.03	2.00 ± 0.08	<0.20
	8	4.74 ± 0.02	3.49 ± 0.06	2.86 ± 0.04	2.34 ± 0.06	<0.20
CP41	6	4.04 ± 0.03	3.12 ± 0.03	1.48 ± 0.07	<0.20	<0.20
	7	4.19 ± 0.03	2.87 ± 0.04	2.62 ± 0.01	2.15 ± 0.02	<0.20
	8	5.48 ± 0.01	3.86 ± 0.02	3.52 ± 0.02	2.57 ± 0.05	1.70 ± 0.07

[a] Means of triplicate experiments (± standard error).

the size and number of particles present in the preparations. Microscopic counts were determined six times for each culture.

The results of the inactivation experiments are shown in Table 1. The 5°C temperature was chosen because it is a temperature where the biocidal activity of chlorine is diminished. These data support the well-established fact that chlorine at lower pH levels, where hypochlorous acid predominates, is more biocidal than at higher pH values, where the less effective hypochlorite ion predominates. In all instances a >3.5-log_{10} reduction occurred after 80 s of exposure. A CT_{99} value of 0.12 mg · min/liter was calculated for a 2-log_{10} (99%) inactivation at pH 6. This pH level was chosen for comparison with previously published inactivation studies conducted under similar conditions, where a CT_{99} of 0.045 mg · min/liter was calculated for *E. coli* (8).

The apparent difference in CT_{99} values between *H. pylori* and *E. coli* was investigated by examining the occurrence of particulate material in the cultures. Microscopic examination revealed large amounts of particulate matter in the *H. pylori* cultures. The particulate matter appeared to be agar debris, doubtlessly transferred in the process of harvesting the organisms from the agar plates. It was observed that cells were associated with the particulate matter, and many of the cells were in an aggregated form. In the size range between 5 and 25 μm, an average of 815 particles per 10 μl was counted in the *H. pylori* cultures. This value contrasts with an average concentration of 6 particles per 10 μl within the same size range for the *E. coli* culture. The difference in the CT_{99} values between *H. pylori* and *E. coli* may well be attributed to the presence of the debris, which provided protection from the disinfectant. Previous studies have shown similar protective mechanisms (1). However, it is important to note that chlorine demand was not a significant factor in the differences observed, since sufficient chlorine was added initially to compensate for the demand of the culture. It is also of interest that calculations based upon data from earlier studies of chlorine inactivation of *E. coli*, under similar conditions where the culture inoculum was removed from agar slants (3), produced a calculated CT value of 0.1 to 0.2 mg · min/liter.

These results indicate that *H. pylori* is sensitive to chlorine. The somewhat increased resistance of *H. pylori*, compared to that of *E. coli* as determined by previous inactivation studies, may be attributed to the method of culture preparation necessitated by the low growth yield of *H. pylori* on artificial media. A survey of disinfection practices in the United States indicated that water utilities provide a median chlorine residual of 1.1 mg/liter and a median exposure time of 45 min to the point of first use in the distribution system (15). This level of chlorination should be more than adequate for inactivating *H. pylori*.

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