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Changes in Virulence of Waterborne Enteropathogens with Chlorine Injury

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We designed experiments to assess the effect of chlorine injury on the virulence of waterborne enteropathogens. Higher chlorine doses (0.9 to 1.5 mg/liter) were necessary to produce injured *Yersinia enterocolitica*, *Salmonella typhimurium*, and *Shigella* spp. than to produce injured enterotoxigenic *Escherichia coli* or coliform bacteria (0.25 to 0.5 mg/liter) in the test system used; 50% lethal dose experiments in which mice were used showed that injured *Y. enterocolitica* cells were 20 times less virulent than uninjured control cells (3,300 and 160 CFU, respectively). This decrease in virulence was not related to reduced attachment to Henle 407 intestinal epithelial cells, but could be related to a loss of HeLa cell invasiveness. In contrast, injured *S. typhimurium* and enterotoxigenic *E. coli* cells lost their ability to attach to Henle cells. These data show that some enteropathogens and coliform bacteria differ in their sensitivities to chlorine injury and that the virulence determinants affected by chlorine may vary from one pathogen to another.

Injury has been defined as the sublethal physiological consequence of exposure to stresses which cause a loss in the ability of microorganisms to grow normally under selective conditions that are satisfactory for untreated cells (3, 17; LeChevallier and McFeters, J. Am. Water Works Assoc., in press). In addition, injury has been described as reversible; that is, under proper conditions of temperature and nutrients, injured organisms can repair the cellular lesion(s) and revert to forms indistinguishable from unstressed cells (3, 17; LeChevallier and McFeters, in press). Coliform bacteria in the environment are known to be stressed by a variety of factors, including chlorine and other disinfectants, heat, freezing, acid mine drainage, transition metals, sunlight, UV irradiation, biological interactions, and possibly other still undefined physicochemical factors (3–5, 8, 9, 11, 17; LeChevallier and McFeters, in press). Recently, new methods have been developed to enumerate injured total and fecal coliform bacteria in drinking water (18, 19).

The question of the significance of injured coliform bacteria in water is a problem that has not been adequately answered. Presumably, conditions that stress coliform bacteria also injure waterborne pathogens, although to our knowledge there are no data to support this hypothesis. Some investigators have examined the virulence of injured foodborne pathogens (6, 12, 30). Sorrells et al. (30) found no difference in the virulence of *Salmonella gallinarum* for 6-week-old chicks after injury by freezing. Fung and Vanden Bosch (12) indicated that *Staphylococcus aureus* regained the ability to produce enterotoxin B after resuscitation in a nonselective medium. However, these results are not directly applicable to the aquatic environment. Walsh and Bissonette (34) studied the attachment of chlorine-injured enterotoxigenic *Escherichia coli* cells to human peripheral leukocytes. These authors concluded that the reduced adhesive ability observed was due to the loss of surface structures resulting from sublethal chlorination.

In this report, we describe the changes in virulence due to chlorine injury of various waterborne pathogens (enterotoxigenic *Escherichia coli*, *Yersinia enterocolitica*, *Salmonella typhimurium* and *Shigella* spp.), as determined by a variety of in vivo and in vitro techniques. In this study we also compared the relative susceptibilities of these pathogens and coliform bacteria to injury by chlorine. Although we found that injured pathogenic bacteria demonstrated reduced virulence, *Y. enterocolitica*, *Salmonella typhimurium*, and the shigellae were much more resistant to injury by chlorine than enterotoxigenic *Escherichia coli* or coliform bacteria.

MATERIALS AND METHODS

Bacterial strains. All of the enteropathogens (except strain E887) were originally isolated from humans. The *Y. enterocolitica* serotypes used included O:3 (strain E752), O:8 (strain E661), O:13 (strain E887; isolated from a monkey), O:20 (strain Em 062), O:21 (strain E750), O:4,32 (strain E759), and O:5,27 (strain E771). The virulence characteristics of most of these isolates have been published previously (26–28). *Salmonella typhimurium* isolates were obtained from the State Laboratory of Hygiene, Madison, Wis., and from H. Lior, Canadian Laboratory Center for Disease Control, Ottawa, Ontario. Two enterotoxigenic *Escherichia coli* cultures were provided by M. Levine, The Center for Vaccine Development, University of Maryland School of Medicine, Baltimore, and a third enterotoxigenic *Escherichia coli* isolate was obtained from R. A. Wilson, *Escherichia coli* Reference Center, Pennsylvania State University, University Park. All three of these isolates were colonization factor antigen positive. *Shigella flexneri* and *Shigella sonnei* were obtained from the University of Washington Clinical Laboratories, Seattle. The *Shigella dysenteriae* isolate used was part of the stock culture collection of Montana State University, Bozeman. The coliform bacteria used were isolated in our laboratory from drinking water by the membrane filter technique (1) and included five strains of *Escherichia coli*, two strains of *Klebsiella oxytoca*, two strains of *Enterobacter cloacae*, one strain of *Enterobacter aerogenes*, and one strain of *Citrobacter freundii*.

All cultures were stored at −70°C in a solution containing 10% peptone and 40% glycerol. For each experiment, cul-

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tures were taken from the frozen stocks and subcultured only once. Therefore, changes in the virulence characteristics of the enteropathogens would not be expected to occur due to repeated passage of the cultures.

Preparation of injured cultures. Bacterial cultures were grown for 24 h in tryptic soy broth without dextrose but supplemented with 1% lactose and 0.3% yeast extract (TLY broth). All cultures except the *Y. enterocolitica* cultures were grown at 35°C; the *Y. enterocolitica* isolates were incubated at 22°C. Cultures were diluted 1×10^6 cells per ml in cold sterile reagent grade water and treated with varying levels of free available chlorine (prepared daily from stock solutions of chlorine bleach [sodium hypochlorite]) (Table 1). The cells were treated with chlorine for 10 to 30 min at 4°C and pH 6.5 to 7.0 in the dark. In this test system, carry-over of nutrients from the TLY broth (1/1,000 dilution) created a chlorine demand of 1.0 ppm (1.0 ng/liter) in 5 min and 1.7 ppm (1.7 ng/liter) within 10 min (measured by the *N,N*-diethyl-*p*-phenylenediamine colorimetric method). Therefore, in most cases injury resulted from a combination of free chlorine and combined chlorine. The system was designed to reproducibly injure bacteria and was not intended to simulate the natural environment. Thus, the chlorine levels used in this study may not be directly compared with those used other studies. Samples were dechlorinated by using sodium thiosulfate (final concentration, 0.01%) (1), which was also added to unchlorinated control cultures.

When high densities of injured cells were needed (approximately 5×10^8 cells per ml), cultures were washed once in cold sterile reagent grade water and treated with chlorine as described above.

The level of chlorine injury was measured by the percent difference in plate counts between a nonselective medium (TLY agar) and a selective medium (TLY agar containing 0.1% deoxycholate) (22). Plates containing TLY agar and TLY agar supplemented with 0.1% deoxycholate were incubated at 35°C for all bacteria except *Salmonella typhimurium* cultures, which were incubated at 43°C.

LD_{50} determinations. Differences in virulence between healthy and injured *Y. enterocolitica* cultures were determined by using 50% lethal dose (LD_{50}) assays and CD-1 mice (Charles River Breeding Laboratory, Inc., Wilminington, Mass.). The cultures were injected intraperitoneally (seven mice per dose). The mice were observed for death for up to 21 days. LD_{50} calculations were made by the method of Reed and Muench (25). Statistical analyses were made by using a generalized linear interactive modeling program on natural logarithimic transformed data (2, 10).

Attachment assays. The procedures used for measuring association between enteropathogens and epithelial cells are described elsewhere (D. A. Schiemann and P. J. Swanz, J. Med. Microbiol., in press). Henle 407 intestinal epithelial cells (ATCC CCL-6 cells) were maintained in Eagle basal medium supplemented with Hanks balanced salts (BME) containing 15% fetal bovine serum, penicillin G (50 IU/ml), and streptomycin (50 μg/ml). The epithelial cells were grown in tissue culture flasks until the monolayers were confluent. On the day before use, fresh medium without antimicrobial agents was provided. Before use, the cells were washed twice with Dulbeccos phosphate-buffered saline without calcium and harvested by scraping the monolayer off the flask wall. The cells were suspended in BME, and the number of viable cells was determined microscopically by trypan blue exclusion. The cell density of the suspension was adjusted to 5.6×10^3 cells per ml by dilution with BME.

Bacteria were grown at room temperature (20°C) for 48 h (*Y. enterocolitica*) or at 35°C for 24 h (*Escherichia coli* and *Salmonella typhimurium*) in 0.13 M phosphate-buffered salts medium containing 20 μCi of [^{35}S]methionine per ml (Schiemann and Swanz, in press). The bacteria were washed in cold sterile reagent grade water and adjusted to a density of 5×10^8 cells per ml. Cells grown in this medium were injured by chlorine treatment as described above. Samples of the bacterial suspension (0.2 ml) were added to 1.8 ml of Henle cells to give a multiplicity of bacteria to epithelial cells of 10^4:1.

The mixture was incubated at 35°C for 5 min. This short incubation time was chosen so that only the process of bacterial attachment was examined, assuming that 5 min was too short a time for engulfment. The contents of the tube were then added to 100 ml of phosphate-buffered saline without calcium on a 5-μm membrane (Nuclepore Corp., Pleasanton, Calif.) that had been pretreated with 50 ml of 1 mM EDTA in 2% peptone (pH 8.2). After filtration under gravity, an additional 100 ml of phosphate-buffered saline without calcium was added to rinse the membrane. Each test was performed in triplicate.

Background control suspensions containing bacteria alone were prepared in a similar manner. The radioactivity of the bacteria was determined by filtering the suspension through a 0.4-μm Nuclepore filter.

Membranes were placed in scintillation vials containing 10 ml of Aquasol (New England Nuclear Corp., Boston, Mass.), and radioactivity was determined by scintillation counting with a Tri-Carb 460 Liquid Scintillation System (Packard Instrument Co., Inc., Downers Grove, Ill.). Counts were converted to disintegrations per minute by reference to an efficiency curve based on ^{14}C. The amount of radioactivity per bacterial cell was calculated to determine the average number of bacteria attached to each Henle cell.

Invasiveness procedures. The procedures used for the HeLa cell infection technique have been described elsewhere (7, 27). HeLa cells (ATCC CCL-2 cells) were maintained in Eagle minimal essential medium supplemented with Earle salts and L-glutamine, 10% fetal bovine serum, 50 IU of penicillin G per ml, and 50 μg of streptomycin per ml. Washed cells were recovered in antibiotic-free BME and adjusted to a density of 2×10^5 cells per ml; 1-ml portions of this suspension were added to plastic tissue culture tubes (16 by 125 mm). Each experiment was performed in triplicate.

Healthy and injured *Y. enterocolitica* O:8 strain E661 cultures were prepared as described above. After chlorine injury, the cells were centrifuged and suspended in BME to a density of 2×10^6 cells per ml, and 1 ml of bacterial suspension was added to a tissue culture tube containing HeLa cells, giving a multiplicity of 10 bacteria per epithelial cell. The mixture was placed on a roller apparatus (Labline Instruments Inc., Melrose Park, N.J.) and incubated at 35°C for 2 h. Resuscitation of injured cells did not occur to an appreciable extent during this time period. After 2 h of infection, 0.1 ml of a gentamicin solution (2.5 mg/ml) was added to each tube, and the preparations were incubated under the same conditions. Since gentamicin does not cross the epithelial cell membrane (7, 27), it killed only the extracellular bacteria and prevented further infection. After this, the tube was centrifuged at $5,500 \times g$ for 10 min at 20°C, and the supernatant was removed; 1-ml of a 1.0% solution of sarcosine in physiological saline was added to lyse the epithelial cells. The contents of the three tubes were combined and plated in triplicate onto TLY agar. Average colony counts were used to calculate the invasiveness index of intracellular bacteria per epithelial cell. Since it was a

concern that sarcosine may have a deleterious effect on intracellular injured *Y. enterocolitica* cells, HeLa cells were also lysed by homogenization at 25,000 rpm (The VirTis Co. Inc., Gardiner, N.Y.).

Microscopic studies. Attachment of enteropathogens was also observed by direct microscopic observation. Cover slips containing Henle cell monolayers were infected with healthy and injured bacteria (5×10^8 cells per ml) for 30 min at 35°C. Infected monolayers were fixed in methanol, stained with May-Grunwald and Giemsa stains, and examined by using a light microscope. At least 50 cells from each preparation were observed.

Samples examined by transmission electron microscopy were prepared by using the HeLa cell invasiveness procedure described above. Samples were fixed by using 3% glutaraldehyde in 0.1 M potassium-sodium phosphate buffer and postfixed by using 2% osmium tertraoxide in potassium-sodium phosphate buffer. Samples were dehydrated with ethanol and embedded in Spurr resin. Thin-sectioned samples were further stained with uranyl acetate and Reynold lead citrate. Samples were examined by using a JEOL model JEM-100 CX scanning-transmission electron microscope.

Materials and reagents. Tryptic soy broth without dextrose and yeast extract were obtained from Difco Laboratories, Detroit, Mich. BME was obtained from Irvine Scientific, Santa Ana, Calif. Minimal essential medium containing Earle salts and Dulbecco phosphate-buffered saline were obtained from GIBCO Laboratories, Grand Island, N.Y. Fetal bovine serum, gentamicin, penicillin G, streptomycin, and sarcosine were obtained from Sigma Chemical Co., St. Louis, Mo.

RESULTS

Chlorine injury. Coliform bacteria serve as indicators of potable water quality by indicating the possible presence of enteric pathogens. It has been suggested that conditions which stress coliform bacteria also injure waterborne pathogens. Because there are no data to support this hypothesis, we investigated the relative susceptibilities of coliform bacteria and pathogens to injury by chlorine. Table 1 shows that coliform bacteria (*Escherichia*, *Enterobacter*, *Citrobacter*, and *Klebsiella*) have the same susceptibility to chlorine as enterotoxigenic *Escherichia coli* (>90% injury produced by 0.25 to 0.5 mg of chlorine per liter), but are much more sensitive than *Y. enterocolitica*, *Salmonella typhimurium*, or the shigellae (injury produced by 0.9 to 1.5 mg of chlorine per liter) in the test system used. Coliform bacteria may be more than 90% injured when *Y. enterocolitica*, *Salmonella typhimurium*, and shigellae are less than 20% stressed. These data indicate that coliform bacteria may be injured (>90%) by chlorine when some pathogens remain unaffected.

TABLE 1. Susceptibilities of coliform bacteria and pathogens to injury by chlorine

Organism	No. of strains tested	Free chlorine concn causing >90% injury (mg/liter)
Coliform bacteria[a]	11	0.38 (0.25-0.50)[b]
Enterotoxigenic *Escherichia coli*	3	0.33 (0.25-0.50)
Y. enterocolitica[c]	7	1.07 (0.50-1.50)
Salmonella typhimurium	7	1.50 (1.00-2.00)
Shigella[d]	3	0.92 (0.50-1.50)

[a] The coliform isolates included five strains of *Escherichia coli*, two strains of *K. oxytoca*, two strains of *Enterobacter cloacae*, one strain of *Enterobacter aerogenes*, and one strain of *C. freundii*.
[b] Values are means. The values in parentheses are ranges.
[c] For a description of the serotypes used, see the text.
[d] The *Shigella* species used included *Shigella dysenteriae*, *Shigella flexneri*, and *Shigella sonnei*.

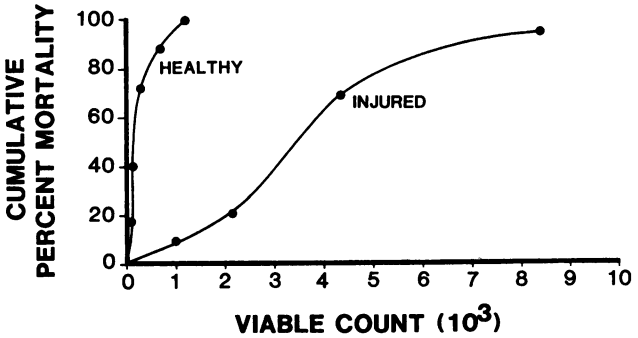

FIG. 1. Comparison of the virulence of healthy and chlorine-injured *Y. enterocolitica* O:8 strain E661 cells in mice. The LD_{50} for healthy bacteria was 160 cells, whereas the LD_{50} for injured organisms was 3,300 cells.

LD_{50} assay. Figure 1 shows a comparison of the virulence of healthy and injured *Y. enterocolitica* O:8 strain E661 cells for mice. Uninjured *Yersinia* cells had an LD_{50} of 160 cells (95% confidence interval, 83 to 271 cells), whereas chlorine-injured *Y. enterocolitica* cells (97% injured) had an LD_{50} that was 20 times higher (3,300 cells; 95% confidence interval, 2,150 to 5,200 cells). The curves were analyzed by a generalized interactive modeling program, which showed that the two LD_{50} values differed significantly ($P << 0.001$). In fact, the deaths caused by the chlorine-injured cells could be attributed to the cells in the population that were not injured (3%; 100 cells). These data indicate that injured *Y. enterocolitica* cells were avirulent in mice.

Y. enterocolitica O:8 contains a virulence plasmid which makes this organism lethal for mice (13, 24). No growth or pinpoint colonies on magnesium oxlate agar indicate calcium dependency mediated by this plasmid (13, 16, 24). Chlorine injury did not alter the calcium dependency of *Y. enterocolitica* O:8. Thus, the decreased virulence of *Y. enterocolitica* could not be attributed to loss of the virulence plasmid.

Attachment assay. We performed experiments to determine which stage of the pathogenic process was affected by chlorine injury. Attachment to an epithelial cell surface is a requirement of all enteropathogenic bacteria. Figure 2 shows that injured *Y. enterocolitica* cells attached to Henle cells just as well as healthy bacteria. Figure 2 also shows that chlorine-killed *Y. enterocolitica* cells attached to epithelial cells at the same level as healthy bacteria.

These data are in contrast to the results obtained with healthy and injured enterotoxigenic *Escherichia coli* and *Salmonella typhimurium* cells (Fig. 3 and 4). Figures 3 and 4 show that with these enteropathogens, increasing levels of chlorine injury correlated with decreasing levels of attachment to Henle cells. In both instances when the pathogens were 90% or more injured, no association was observed between bacteria and epithelial cells. Clearly, the virulence determinants affected by chlorine injury in these two pathogens differ from those observed with *Y. enterocolitica*.

An examination of stained monolayers infected with healthy and injured enterotoxigenic *Escherichia coli* and *Salmonella typhimurium* cells confirmed the conclusions

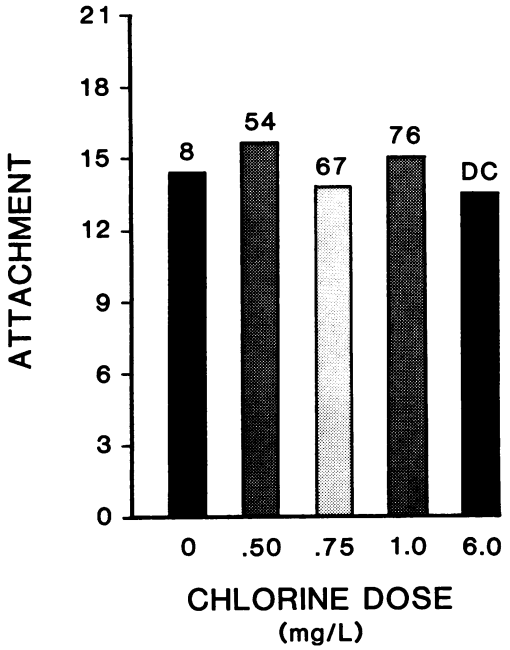

FIG. 2. Attachment of *Y. enterocolitica* cells to Henle 407 intestinal epithelial cells with increasing chlorine doses. Attachment was measured as the average number of bacteria per Henle cell. The numbers at the tops of the columns indicate percent injury. DC, Dead cells.

described above (Fig. 5). At least 50 cells were examined from each preparation. Uninjured cells attached at an average level of more than 100 bacteria per epithelial cell (in many cases too numerous to count), whereas injured enterotoxigenic *Escherichia coli* and *Salmonella typhimurium* cells attached at an average level of 17 and 10 bacteria per Henle cell, respectively.

Invasiveness assay. The second step required for virulence of *Y. enterocolitica* is the ability to invade epithelial cells. The levels of invasiveness of healthy and injured *Y. enterocolitica* O:8 strain E661 cells are compared in Fig. 6. As determined by the roller tube procedure, healthy *Y. enterocolitica* cells averaged 1.15 bacteria per HeLa cell, whereas injured cells showed an invasiveness index of only 0.01 bacterium per epithelial cell. Gentamicin effectively killed extracellular bacteria, reducing background counts by more than 5 logs.

Differences in the methods used to disrupt epithelial cells did not affect the results of the experiment. Lysis of HeLa cells infected with injured *Y. enterocolitica* cells by homogenization and with sarcosine yielded intracellular bacteria at 1.4 and 0.4%, respectively, of the healthy cell level.

An examination of HeLa cells by transmission electron microscopy showed that more than 90% of the cells infected by uninjured *Y. enterocolitica* cells contained intracellular bacteria, whereas 82% of the preparations infected with injured cells (73% injury) lacked evidence of intracellular organisms (Fig. 7).

During the injury procedure some bacteria died as a result of exposure to chlorine. Decreases in viability were as high as 50% in some experiments. It was a concern that these dead cells might inhibit the infectivity of the injured population. Experiments were conducted with varying ratios of chlorine-killed and uninjured *Y. enterocolitica* cells (0.8:1, 1.6:1, and 8:1). Our results indicated that chlorine-killed cells did not interfere with the invasiveness of healthy cells. The invasiveness indexes for this experiment were 6.6 for the uninjured control cells and 5.8, 6.8, and 7.1 for preparations containing live and dead cells at ratios of 1:0.8, 1:1.6, and 1:8.0, respectively.

The level of invasiveness of *Y. enterocolitica* cells resuscitated in TLY broth after chlorine injury was also investigated. Cells were injured (99%) as described above and then resuscitated in TLY broth for 3 h at 20°C. The results of these experiments indicated that after 3 h of incubation *Y. enterocolitica* repaired the chlorine-induced lesions and the

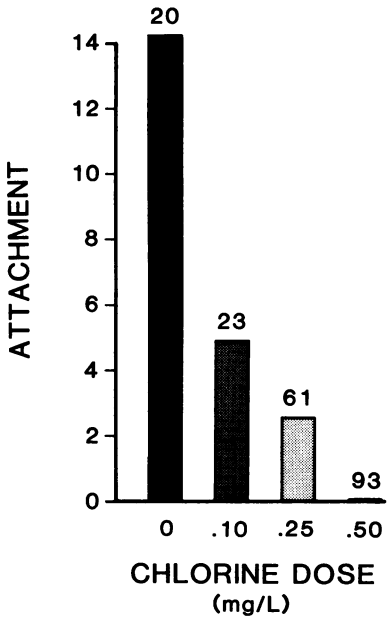

FIG. 3. Attachment of enterotoxigenic *Escherichia coli* cells to Henle 407 intestinal epithelial cells with increasing chlorine doses. Attachment was measured as the average number of bacteria per Henle cell. The number at the tops of the columns indicate percent injury.

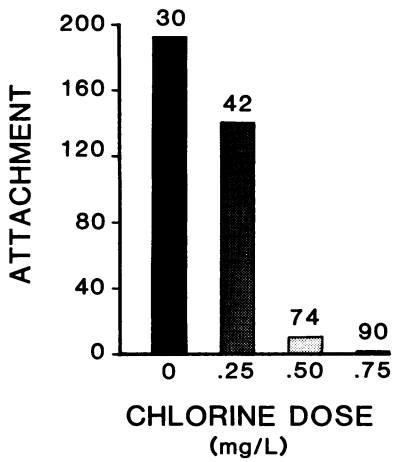

FIG. 4. Attachment of *Salmonella typhimurium* to Henle 407 intestinal epithelial cells with increasing chlorine doses. Attachment was measured as the average number of bacteria per Henle cell. The numbers at the tops of the columns indicate percent injury.

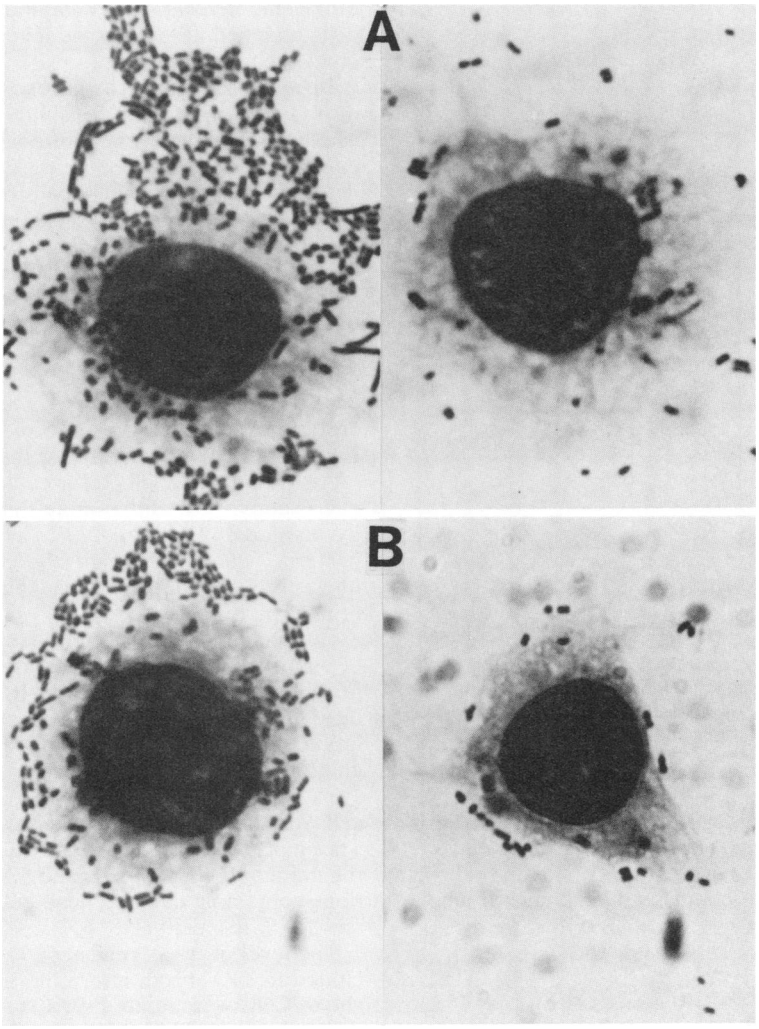

FIG. 5. (A) Photomicrographs of healthy (left) and injured (right) enterotoxigenic *Escherichia coli* cells attached to Henle 407 intestinal epithelial cells. (B) Photomicrographs of healthy (left) and injured (right) *Salmonella typhimurium* cells attached to Henle cells.

cells regained their infectivity. The invasiveness indexes for healthy and injured cells were 0.31 and 0.32, respectively.

DISCUSSION

The bacterial quality of potable water is presently assessed by an indirect method in which indicator bacteria collectively known as the coliform group are used. The premise underlying this test is that coliform bacteria indicate the potential presence of pathogenic microorganisms. Recently, the use of coliform bacteria as indicators of potable water quality has been questioned, in part because coliform bacteria can become injured due to stresses in the aquatic environment and subsequently fail to grow on selective media commonly used for their enumeration. Various reports (4, 18, 19; LeChevallier and McFeters, in press) have indicated that as many as 90% of the indicator bacteria found in potable and surface waters may not be recovered by standard techniques.

Presumably, conditions that stress coliform bacteria also injure waterborne pathogens, although there are no data to support this hypothesis. The results of this investigation indicate that coliform bacteria are more susceptible to injury

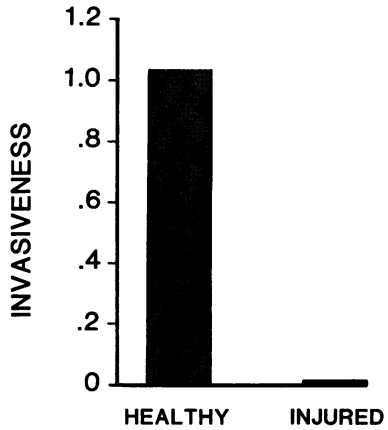

FIG. 6. Levels of invasiveness of healthy and injured *Y. enterocolitica* cells into HeLa cells. Invasiveness was measured as the average number of intracellular bacteria per HeLa cell.

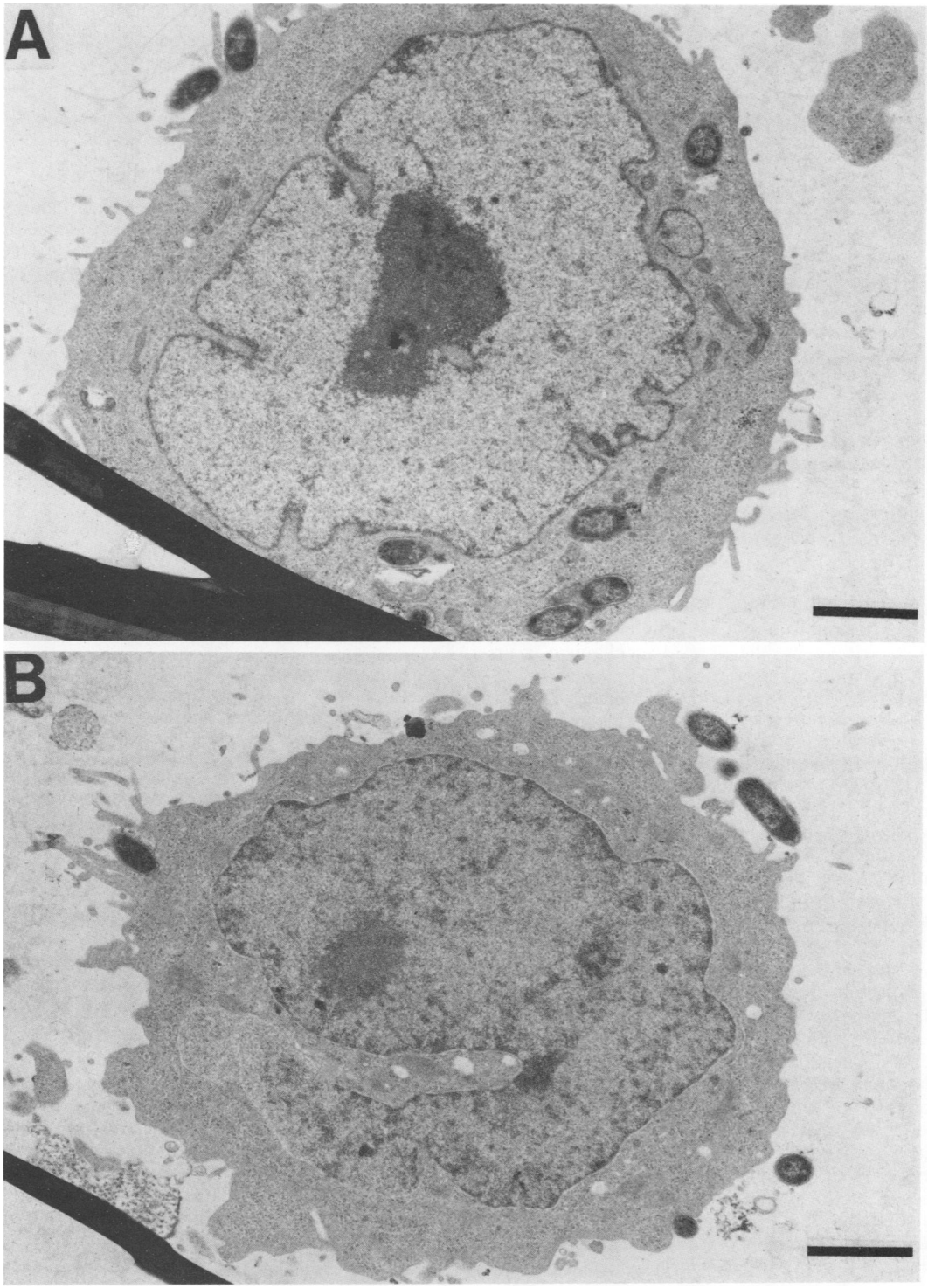

FIG. 7. Transmission electron micrographs of HeLa cells infected with healthy (A) and injured (B) *Y. enterocolitica* cells. Bars = 2 μm.

by chlorine than *Y. enterocolitica*, *Salmonella typhimurium*, and shigellae. The indicator bacteria may show more than 90% injury when the pathogens are not affected. The susceptibility of coliform bacteria to chlorine injury did correlate with the sensitivity of enterotoxigenic *Escherichia coli* to this disinfectant. An important conclusion from these results is that coliform bacteria and various potential waterborne pathogenic microorganisms may differ in their sensitivities to chlorine injury. Further research on other enteric pathogens and different aquatic stresses must be done before a comprehensive picture is possible.

Our results indicate that chlorine-injured *Y. enterocolitica* cells are avirulent when they are tested by the mouse LD_{50} assay. The deaths observed with an injured suspension can

be attributed to the small percentage of uninjured healthy bacteria remaining in the population. These results are in contrast to those reported by other investigators (6, 12, 30). Injuries caused by heat (6), freezing (30), and freeze-drying (12) did not significantly reduce the virulence of foodborne pathogens. However, these types of stresses may cause different physiological responses than those produced by chlorine injury.

The ability of injured *Y. enterocolitica* cells to attach to epithelial cells when injured enterotoxigenic *Escherichia coli* and *Salmonella typhimurium* cells had lost this capacity indicates that the virulence determinants affected by chlorine may vary from one pathogen to another. Our results support the conclusions of Walsh and Bissonnette (34), who reported that chlorine-injured enterotoxigenic *Escherichia coli* cells lost the ability to attach to peripheral leukocytes. It is possible that chlorine damaged the fimbriae responsible for the interaction between enterotoxigenic *Escherichia coli* and *Salmonella typhimurium* cells and epithelial cells. Our data (Fig. 3 and 4) show considerable loss of attachment by enterotoxigenic *Escherichia coli* and *Salmonella typhimurium* cells at low chlorine doses when injury was not observed by differential plate counts. These data might be explained if chlorine damaged the fimbriae before it affects the cell membrane (35). Since *Y. enterocolitica* cells do not produce fimbriae when they are grown for short periods at 22°C (20, 28, 32; Schiemann and Swanz, in press), attachment of these pathogens to epithelial cells is mediated in a different manner. However, the precise mechanism is unknown.

Injury caused by sublethal exposure to chlorine did affect the ability of *Y. enterocolitica* to invade HeLa cells in vitro. Since tissue culture invasiveness has been characterized as a virulence requirement for this organism (7, 20, 23, 26, 28, 31, 32), it is likely that the lack of invasiveness observed in vitro is responsible for the decreased virulence observed in vivo. Additional experiments are in progress to verify this hypothesis.

The physiological reason why injured *Y. enterocolitica* cells lose the ability to invade epithelial cells is an intriguing question with important implications concerning the pathogenicity of this organism. Hale et al. (15) indicated that enteroinvasive shigellae do not act as inert particles since nonviable or avirulent shigellae do not initiate infection of epithelial cells. These investigators indicated that physiological and synthetic functions of the bacterium are required. Other researchers have shown that some specific interaction is necessary between the bacteria and the epithelial cells (22, 29). It is thought that whether the attached bacteria are ingested depends on bacterial surface properties, such as hydrophobicity and electric charge (22, 29). Many investigators have previously shown that chlorine causes damage to cell membranes of coliform bacteria (5, 14, 33, 35). Experiments have also shown that respiration, glucose transport, and ATP levels all decrease in injured coliform populations (5, 14, 33). Electron microscopy of injured cells has demonstrated morphological changes in the cell membrane (35). Whether these changes also occur in *Y. enterocolitica* and whether these changes are responsible for an inability to invade epithelial cells remain to be seen. Experiments to answer these important questions are in progress.

In summary, we have shown that *Y. enterocolitica* cells injured by sublethal concentrations of chlorine have reduced virulence when they are tested by using the mouse LD_{50} assay and that this decreased virulence could be related to a loss of HeLa cell invasiveness. *Y. enterocolitica*, *Salmonella typhimurium*, and shigellae showed lower susceptibilities to injury by chlorine than did enterotoxigenic *Escherichia coli* and various members of the coliform group. However, important questions remain regarding other aquatic stresses, the physiology of chlorine injury with other pathogens, and the mechanisms of reduced virulence.

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LITERATURE CITED

1. **American Public Health Association.** 1980. Standard methods for the examination of water and wastewater, 15th ed. American Public Health Association, Washington, D.C.
2. **Baker, R. J., and J. A. Nelder.** 1978. The GLIM system—generalized linear interactive modeling. The Numerical Algorithm Group, Oxford, England.
3. **Beuchat, L. R.** 1978. Injury and repair of gram-negative bacteria, with special considerations of the involvement of the cytoplasmic membrane. Adv. Appl. Microbiol. **23**:219–243.
4. **Bissonnette, G. K., J. J. Jezeski, G. A. McFeters, and D. G. Stuart.** 1975. Influence of environmental stresses on enumeration of indicator bacteria from natural waters. Appl. Environ. Microbiol. **29**:186–194.
5. **Camper, A. K., and G. A. McFeters.** 1979. Chlorine injury and the enumeration of waterborne coliform bacteria. Appl. Environ. Microbiol. **37**:633–641.
6. **Collins-Thompson, D. F., A. Hurst, and H. Kruse.** 1973. Synthesis of enterotoxin B by *Staphylococcus aureus* after recovery from heat injury. Can. J. Microbiol. **19**:1463–1468.
7. **Devinish, J. A., and D. A. Schiemann.** 1981. HeLa cell infection by *Yersinia enterocolitica*: evidence for lack of intracellular multiplication and development of a new procedure for quantitative expression of infectivity. Infect. Immun. **32**:48–55.
8. **Domek, M. J., M. W. LeChevallier, S. C. Cameron, and G. A. McFeters.** 1984. Evidence for the role of copper in the injury process of coliforms in drinking water. Appl. Environ. Microbiol. **48**:289–293.
9. **Double, M. D., and G. K. Bissonnette.** 1980. Enumeration of coliforms from streams containing acid mine water. J. Water Pollut. Control Fed. **52**:1947–1952.
10. **Finney, D. J.** 1978. Statistical methods in biological assay. MacMillin Publishing Co., Inc., New York.
11. **Fujioka, R. S., H. H. Hashimoto, E. B. Siwak, and H. F. Reginald.** 1981. Effect of sunlight on survival of indicator bacteria in seawater. Appl. Environ. Microbiol. **41**:690–696.
12. **Fung, D. Y., and L. L. Vanden Bosch.** 1975. Repair, growth, and enterotoxigensis of *Staphylococcus aureus* S-6 injured by freeze-drying. J. Milk Food Technol. **38**:212–218.
13. **Gemski, P., J. R. Lazere, and T. Casey.** 1980. Plasmid associated with pathogenicity and calcium dependency of *Yersinia enterocolitica*. Infect. Immun. **27**:682–685.
14. **Haas, C. W., and R. S. Engelbrecht.** 1980. Physiological alterations of vegetative microorganisms resulting from chlorination. J. Water Pollut. Control Fed. **52**:1976–1989.
15. **Hale, T. L., and P. F. Bonventre.** 1979. Shigella infection of Henle intestinal epithelial cells: role of the bacterium. Infect. Immun. **24**:879–886.
16. **Higuchi, K., and J. L. Smith.** 1961. Studies on the nutrition and physiology of *Pasteurella pestis*. IV. A differential plating medium for the estimation of the mutation rate to avirulence. J. Bacteriol. **81**:605–608.
17. **Hoadley, A. W., and C. M. Cheng.** 1974. Recovery of indicator bacteria on selective media. J. Appl. Bacteriol. **37**:45–57.

18. **LeChevallier, M. W., S. C. Cameron, and G. A. McFeters.** 1983. New medium for the improved recovery of coliform bacteria from drinking water. Appl. Environ. Microbiol. **45**:484–492.
19. **LeChevallier, M. W., P. E. Jakanoski, A. K. Camper, and G. A. McFeters.** 1984. Evaluation of m-T7 agar as a fecal coliform medium. Appl. Environ. Microbiol. **48**:371–375.
20. **Lee, W. H., P. P. McGrath, P. H. Carter, and E. L. Eide.** 1977. The ability of some *Yersinia enterocolitica* strains to invade HeLa cells. Can. J. Microbiol. **23**:1714–1722.
21. **McFeters, G. A., S. C. Cameron, and M. W. LeChevallier.** 1982. Influence of diluents, media, and membrane filters on the detection of injured waterborne bacteria. Appl. Environ. Microbiol. **43**:97–103.
22. **Ohmann, L., J. Hed, and O. Stendahl.** 1982. Interaction between human polymorphonuclear leukocytes and two different strains of type 1 fimbriae-bearing *Escherichia coli*. J. Infect. Dis. **146**:751–757.
23. **Pedersen, K. B., S. Winblad, and V. Bitsch.** 1979. Studies on the interaction between different O-serotypes of *Yersinia enterocolitica* and HeLa cells. Acta Pathol. Microbiol. Scand. **87**:141–145.
24. **Portnoy, D. A., S. L. Moseley, and S. Falkow.** 1981. Characterization of plasmids and plasmid-associated determinants of *Yersinia enterocolitica* pathogenesis. Infect. Immun. **31**:775–782.
25. **Reed, L. J., and H. Muench.** 1938. A simple method of estimating fifty percent endpoints. J. Hyg. **27**:493–497.
26. **Schiemann, D. A., and J. A. Devenish.** 1980. Virulence of *Yersinia enterocolitica* determined by lethality in Mongolian gerbils and by the Sereny test. Infect. Immun. **29**:500–506.
27. **Schiemann, D. A., and J. A. Devenish.** 1982. Relationship of HeLa cell infectivity to biochemical, serological, and virulence characteristics of *Yersinia enterocolitica*. Infect. Immun. **35**:497–506.
28. **Schiemann, D. A., J. A. Devenish, and S. Toma.** 1981. Characteristics of virulence in human isolates of *Yersinia enterocolitica*. Infect. Immun. **32**:400–403.
29. **Sharon, N.** 1984. Surface carbohydrates and surface lectins and recognition determinants in phagocytosis. Immun. Today **5**:143–147.
30. **Sorrells, K. M., M. L. Speck, and J. A. Warren.** 1970. Pathogenicity of *Salmonella gallinarum* after metabolic injury by freezing. Appl. Microbiol. **19**:39–43.
31. **Une, T.** 1977. Studies on the pathogenicity of *Yersinia enterocolitica*. II. Interaction with cultured cells *in vitro*. Microbiol. Immunol. **21**:365–377.
32. **Une, T., H. Zen-Yoji, T. Maruyama, and Y. Yanagawa.** 1977. Correlation between epithelial cell infectivity *in vitro* and O-antigen groups of *Yersinia enterocolitica*. Microbiol. Immunol. **21**:727–729.
33. **Venkobachar, C., L. Iyengar, and A. V. S. P. Rao.** 1977. Mechanism of disinfection: effect of chlorine on cell membrane functions. Water Res. **11**:727–729.
34. **Walsh, S. M., and G. K. Bissonette.** 1983. Chlorine-induced damage to surface adhesins during sublethal injury of enterotoxigenic *Escherichia coli*. Appl. Environ. Microbiol. **45**:1060–1065.
35. **Zaske, S. K., W. S. Dockins, and G. A. McFeters.** 1980. Cell envelope damage in *Escherichia coli* caused by short-term stress in water. Appl. Environ. Microbiol. **41**:386–390.