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Use of Autoradiography To Assess Viability of *Helicobacter pylori* in Water

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Autoradiographic methods have been developed to detect metabolic activity of viable but nonculturable cells of *Helicobacter pylori* in water. Four strains of *H. pylori* were studied by using microcosms containing suspensions of 72-h cultures in water. The suspensions of aged, nonculturable cells of *H. pylori* were incubated with [^{3}H]thymidine for 24 to 72 h, after which the cell suspensions were exposed to Kodak NTB2 emulsion for 3 to 28 days. Each sample was processed with three separate controls to rule out false-positive reactions. The organism remains viable and culturable under these conditions for up to 48 h and, in some cases, 20 to 30 days, depending on physical conditions of the environment. We found that temperature was a significant ($P \leq 0.01$) environmental factor associated with the viability of *H. pylori* cells in water. Autoradiographs of tritium-labeled cells of *H. pylori* revealed aggregations of silver grains associated with uptake by *H. pylori* of radiolabelled substrate. Findings based on the autoradiographic approach give strong evidence supporting the hypothesis that there is a waterborne route of infection for *H. pylori*. The possibility that *H. pylori* may persist in water in a metabolically active stage but not actively growing and dividing is intriguing and relevant to public health concerns.

Helicobacter pylori (formerly *Campylobacter pylori* [5]) is a fastidious, microaerophilic, gram-negative bacterium associated with type B gastritis (1) or peptic ulcer disease (13) or both. Recently published data suggest that infection with *H. pylori* may be a risk factor for gastric carcinoma (11, 12, 18). Although the evidence for *H. pylori* as a human pathogen is compelling, the mode of transmission remains unclear. Fecal-oral and oral-oral routes of transmission have been suggested (8). The possibility that *H. pylori* infection is a zoonosis has been reported (19, 20), but *H. pylori* has not been isolated from sources outside the human gastrointestinal tract and no animal or environmental reservoirs have been identified. Recently published epidemiological data showed that the prevalence of infection in children was associated with the source of their drinking water. This association between consumption of water and *H. pylori* infection suggests that under appropriate circumstances *H. pylori* may be transmitted by a waterborne route (8). Assuming *H. pylori* may be an agent of waterborne disease, mechanisms of survival of the organism in natural water need to be determined. Research in our laboratory over the past several years has shown that *H. pylori* can survive in water microcosms in a viable but dormant form (16, 17). Similar observations have been reported by West et al. (21). In this report, we present results of experiments employing autoradiography that demonstrate metabolic activity of viable but nonculturable cells of *H. pylori* exposed to water under various temperature regimens.

H. pylori strains from the National Collection of Type Culture (London, England), RSB6, TX30A, 95E, and 26695, were used in these studies.

Inocula were prepared by harvesting cells from 72-h cultures of *H. pylori* on blood agar plates, using sterile disposable polystyrene loops, and suspending the cells in a 50-ml polypropylene, sterile, disposable centrifuge tube (nontoxic ethylene oxide residue; Falcon Plastics, Lincoln Park, N.J.) containing 15 ml of autoclaved river water or distilled water. The cell suspensions were used to seed individual, acid-washed, glass bottles (Corning Glassware Inc., Corning, N.Y.) containing appropriate amounts of sterile river water or distilled water. In a separate set of experiments, suspensions of *H. pylori* were prepared in a series of sterile 50-ml disposable centrifuge tubes containing 20 ml of sterile distilled water. Cells were harvested by centrifugation at 8,000 × *g* for 15 min, washed three times in sterile distilled water, and resuspended in 20 ml of sterile distilled water. All bottles and tubes containing inocula, as well as controls, were incubated at 4, 15, 22, or 37°C.

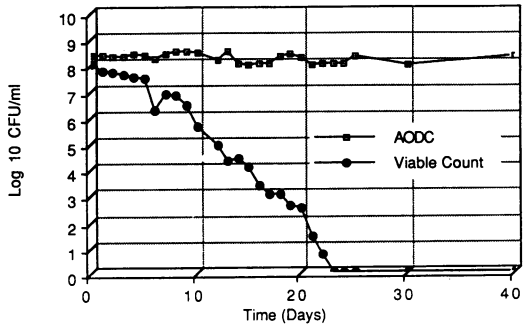

FIG. 1. Survival of *H. pylori* 95E in sterile river water incubated at 4°C.

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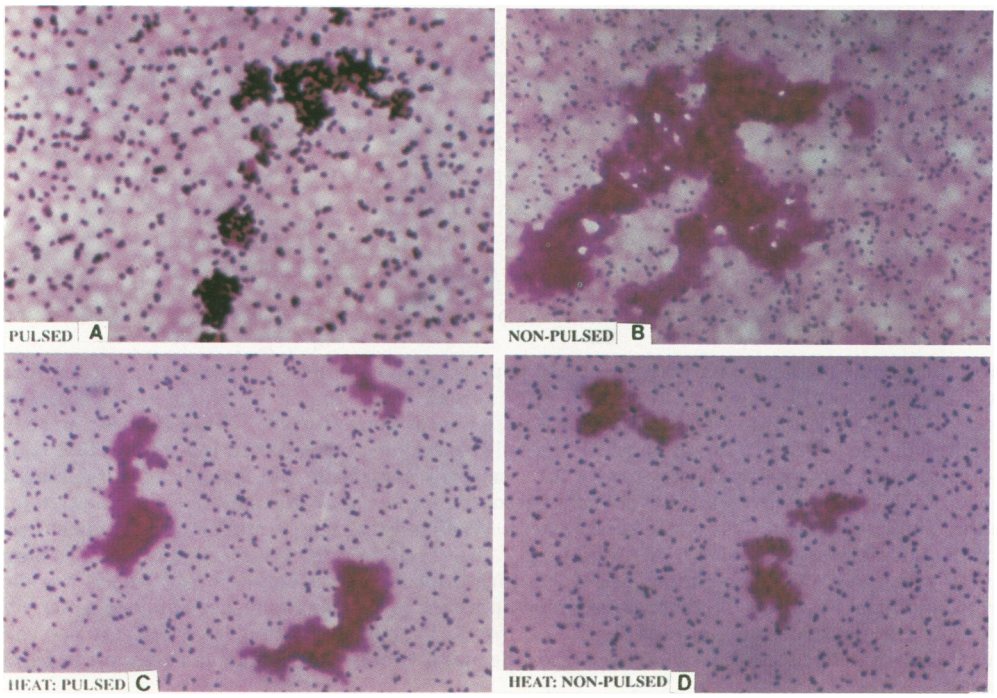

FIG. 2. Microautoradiographs of tritium-labeled cells of *H. pylori* 95E suspended in distilled water and stored at 4°C for 26 months. The photographs show the spectrum of unlabeled and labeled cells (with associated silver grains) of *H. pylori*. (A) Cells pulsed with [^{3}H]thymidine; (B) cells not pulsed with [^{3}H]thymidine; (C) cells heated first and then pulsed with [^{3}H]thymidine; (D) cells heated but not pulsed with [^{3}H]thymidine.

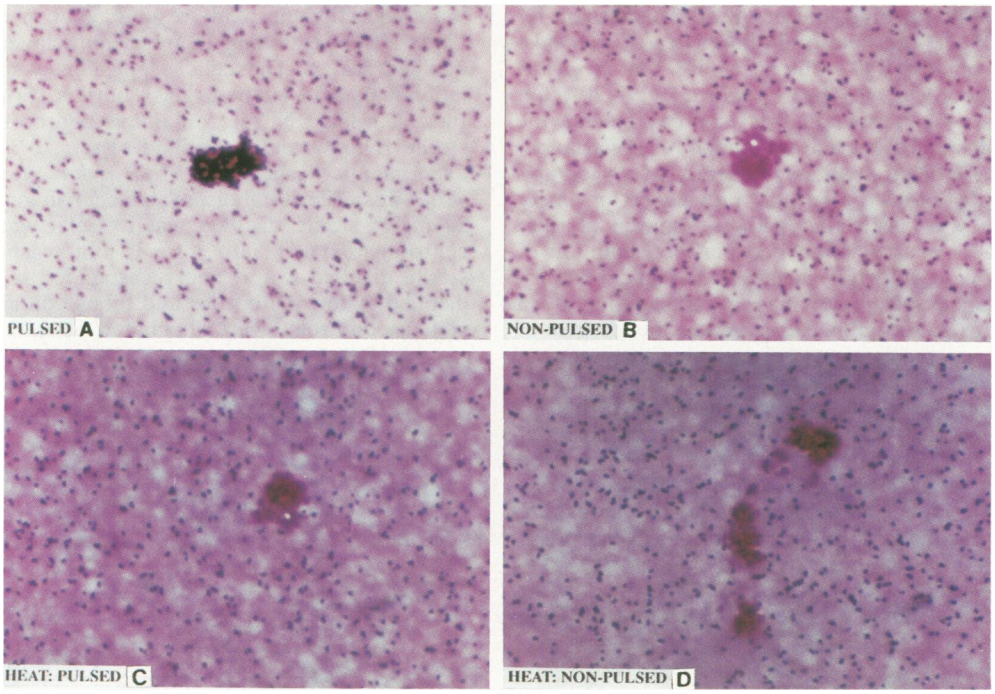

FIG. 3. Microautoradiographs of tritium-labeled cells of *H. pylori* 95E suspended in river water and stored at 4°C for 26 months. The photographs show the spectrum of unlabeled and labeled cells (with associated silver strains) of *H. pylori*. (A) Cells pulsed with [^{3}H]thymidine; (B) cells not pulsed with [^{3}H]thymidine; (C) cells heated first and then pulsed with [^{3}H]thymidine; (D) cells heated but not pulsed with [^{3}H]thymidine.

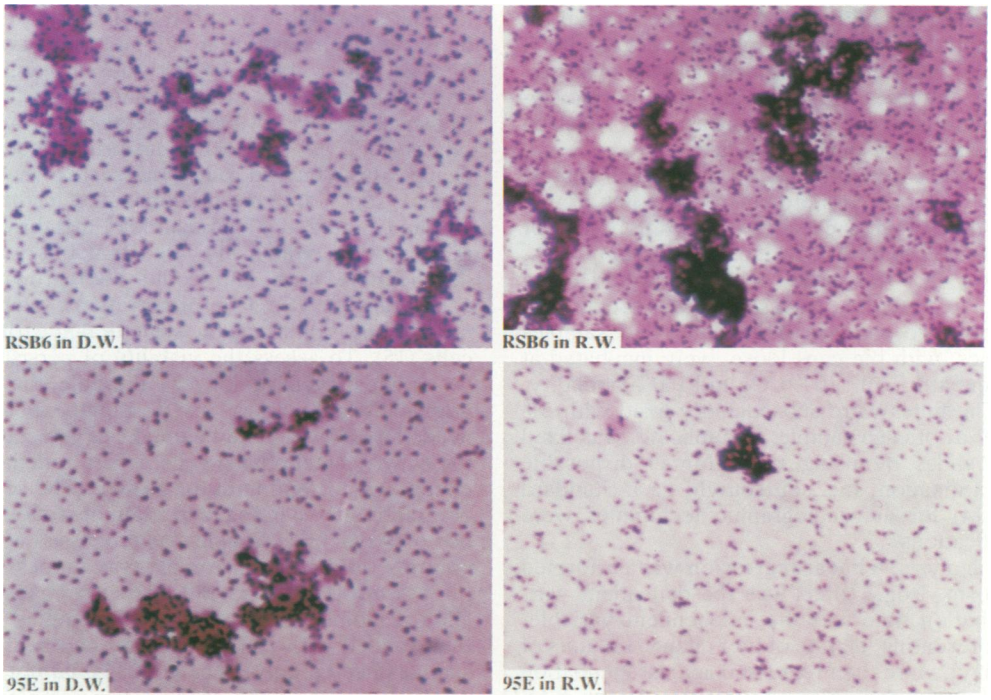

FIG. 4. Microautoradiographs of tritium-labeled cells of *H. pylori* RSB6 and 95E suspended in river water (R.W.) and in distilled water (D.W.) and stored at 4°C for 26 months. The photographs show a comparison of silver grain densities on the cell clumps.

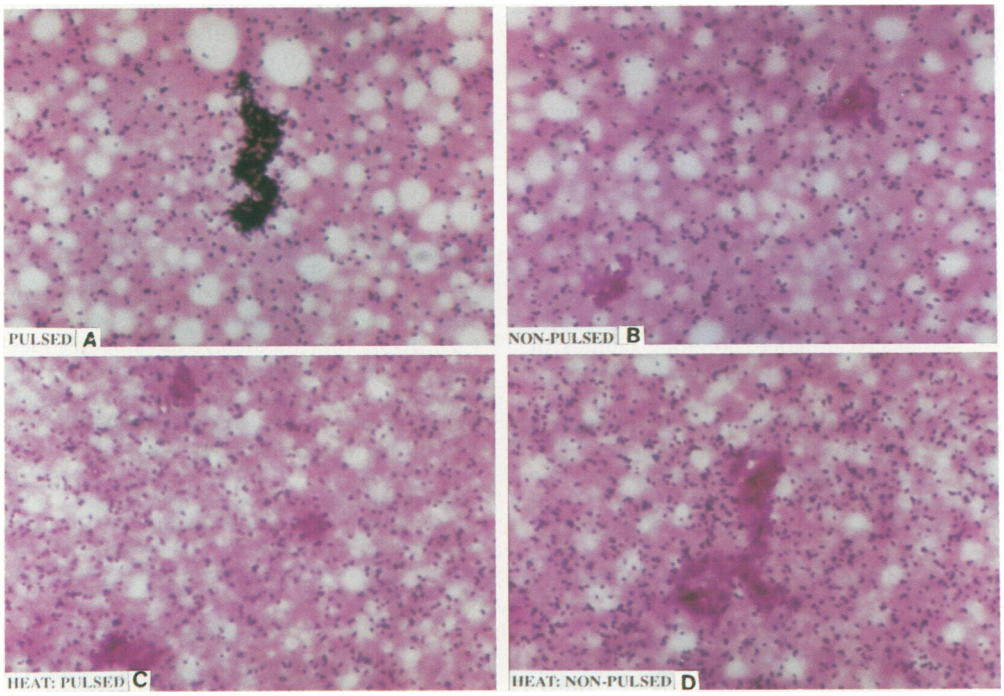

FIG. 5. Microautoradiographs of tritium-labeled cells of *H. pylori* TX30A suspended in river water and stored at 4°C for 38 months. The photographs show the spectrum of unlabeled and labeled cells (with associated silver grains) of *H. pylori*. (A) Cells pulsed with [^{3}H]thymidine; (B) cells not pulsed with [^{3}H]thymidine; (C) cells heated first and then pulsed with [^{3}H]thymidine; (D) cells heated but not pulsed with [^{3}H]thymidine.

Samples from each of the bottles and tubes were tested for viable count by plating on blood agar. Acridine orange direct counts (AODC) (6) were also performed, as was measurement of enzymatic activities and observations of morphological changes, monitored by phase-contrast and electron microscopy, at appropriate time intervals.

For substrate uptake experiments, 10 ml of each sample was pipetted into each of four sterile disposable polypropylene tubes. Two tubes from each sample set were boiled for 2 min. [^{3}H]thymidine (86 to 90 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) was added to a boiled and to an unboiled tube (3.3 μCi/ml); i.e., each sample was prepared in quadruplicate with two samples boiled and two samples unboiled. All tubes, with the caps loose, were incubated at 37°C in a CO_2 incubator, with 10% CO_2 and 100% humidity, for 24 to 72 h. The tubes were subsequently fixed with 2% formalin, and 100 μl of each sample, in duplicate, was fixed on acid-cleaned slides by using a cytofunnel disposable sample chamber (Shandon, Inc., Pittsburgh, Pa.). Standard methods (3, 4, 14) for autoradiographic techniques were used, with modifications.

For statistical analysis, a linear regression was performed for each microcosm. Log_{10} (titer) was the dependent variable, while incubation time was the independent variable. These slopes, representing the rate of titer change [in units of $\log_{10}$ [titer] per day], were then regressed against incubation temperature. All computations were performed with SAS programs (SAS Institute, Cary, N.C.).

Enumeration of *H. pylori* cells suspended in river water and distilled water and incubated at different temperatures for variable periods of time was done by both viable count (plate count) and direct total count (AODC). Figure 1, which represents the results of three repeated experiments, shows that the viable count declined rapidly from 10^8 to 0 CFU/ml between days 20 and 25 in a microcosm at 4°C. In contrast, the total direct count, employing AODC, remained constant for the duration of the experiment. AODC was performed at selected time intervals for more than 2 years (data not shown). Ongoing to date, the total number of cells do not show any significant reduction. Samples which were incubated at higher temperatures, e.g., 22 and 37°C, however, lost culturability within 24 to 48 h, even though the AODC remained stable. Although incubation at 15°C resulted in a drastic decline of CFU per milliliter, the organism remained culturable for up to 10 to 15 days, while the AODC of all samples remained essentially constant, at the initial cell concentration used for inoculation. There were some fluctuations in AODC due to clumping of cells rather than fluctuation in actual cell number; counting clumps of cells is not easy, and the results may not always be accurate.

Survival and culturability of *H. pylori* in different aquatic environments have been reported by other investigators (21, 22). The results of our experiments showed that elevated temperature of incubation of samples resulted in a loss of culturability within 48 h and loss of metabolic viability (data not shown). Whereas at 4°C we saw a change of -0.31 $\log_{10}$ (viable count) per day, at 15, 22, and 37°C these rates of change were -0.49, -2.57, and -4.14 $\log_{10}$ (viable count) per day, respectively. We found that temperature was a significant ($P \leq 0.01$) environmental factor associated with the viability of *H. pylori* cells in water. It is clearly understood that sterile river water and distilled water do not reflect natural environments, in which predation by protozoan species and competition with natural populations of microorganisms can occur. However, as a first step, it is vital that the mere possibility of survival of *H. pylori* in natural water be tested. Demonstration of long-term survival of *H. pylori* in river water, distilled water, and drinking water is a very important first step in establishing a waterborne route of transmission of *H. pylori*. As described previously by us and others (2, 7, 9, 10), the organism undergoes morphological changes from curved rods to horseshoe-shaped and coccoid forms. Coccoid forms predominated in the water samples (10) and exhibited a high degree of membrane and cytoplasmic integrity, which indicated that this was not simply a degenerative form and could be regrown under special conditions (2, 16). Our observations, employing electron microscopy, confirmed the structural integrity of the coccoid cells (15). To assess the viability of persisting forms of *H. pylori* which were no longer culturable, the microautoradiographic technique was used. Long exposure times were needed to allow thymidine uptake in detectable quantities by the slowly metabolizing cells, which remained viable under conditions not optimum for growth. The series of photomicrographs shown in Fig. 2 to 5 demonstrate uptake. It should be noted that each sample was processed with its own set of controls (in fact, each sample was processed with a set of three controls), i.e., nonpulsed (cell samples not treated with [^{3}H]thymidine), heat pulsed (cell samples heated first and then treated with [^{3}H]thymidine), and heat nonpulsed (cell samples heated only). The controls did not show any false-positive results. None of the seven samples showing metabolically active cells yielded false-positives in controls (Fig. 2 to 5).

As described above, the significant environmental parameter associated with viability of *H. pylori* cells in water was temperature. *H. pylori* remained viable, even though nonculturable, at temperatures of 4 and 15°C. A temperature of 15°C was especially important, since many rivers have water temperatures of ca. 15°C most of the year in temperature climates. The recent publication by Klein et al. (8) on the prevalence of infection in children and association of infection with source of drinking water and waterborne spread of *H. pylori*, viewed in light of our findings with autoradiography, offers confirmation that a waterborne route for *H. pylori* may exist.

To establish an environmental reservoir for *H. pylori* requires an understanding of the ecology of this organism, using carefully defined and controlled conditions in the laboratory as well as in the natural environment. The possibility that *H. pylori* may persist in water in a viable but nonculturable form is intriguing and certainly should prove of public health significance.

H. pylori appears to have developed a mechanism to resist adverse effects of the aquatic environment by entering into a persistent or dormant form and stays viable for a very long time. The persistent form of *H. pylori* may play an important role in the transmission of disease.

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