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Pretreatment with Urea-Hydrochloric Acid Enhances the Isolation of *Helicobacter pylori* from Contaminated Specimens

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Human saliva seeded with *H. pylori* was incubated in urea-HCl and then cultured on nonselective media. Pretreatment with 0.06 N HCl–0.08 M urea for 5 min at 37°C resulted in reproducible isolation of *H. pylori*, even at low inocula ($\leq 10^2$ CFU/ml of saliva), despite the presence of large numbers of contaminating organisms.

The transmission route and source of *Helicobacter pylori* infection remain unclear. The presence of *H. pylori* DNA in the oral cavity, feces, and water has been demonstrated using PCR (7, 10, 17, 21), but the culture of *H. pylori* from these specimens using established methods is quite difficult (1, 2, 6, 18, 22). These specimens may contain low numbers of *H. pylori* organisms (20), which are likely to be overgrown by more abundant populations of rapidly growing competing microorganisms even on selective media. Urease is found in the cytoplasm and on the membrane of *H. pylori* cells (5, 8). Compared to other urease-positive microorganisms, *H. pylori* produces larger quantities of highly active urease (5, 9, 19). Urease hydrolyzes urea, creating a basic "ammonia cloud" around the bacteria, thereby allowing *H. pylori* to survive at low pH in the presence of urea under conditions similar to those in the stomach (3, 12, 16). The aim of this study was to develop a new method using short-duration exposures to hydrochloric acid (HCl) plus urea to facilitate the isolation of *H. pylori* from highly contaminated specimens.

Local institutional review board approval for specimen collection was obtained, and patients gave informed written consent. Saliva was obtained from an *H. pylori*-negative volunteer. Primary *H. pylori* cultures were obtained from patients undergoing upper endoscopy. *H. pylori* type strain ATCC 43504 was grown on heart infusion agar with 5% rabbit blood (BBL, Cockeysville, Md.) for 48 h at 37°C under microaerobic conditions (85% N_2, 10% CO_2, 5% O_2) and then suspended in normal saline for the following assays. First, pure cultures of *H. pylori* were tested to determine survival in various urea-HCl concentration ranges. Ten microliters of the diluted suspension ($\sim 10^5$ CFU of *H. pylori*) was incubated with 5 μl of urea and 10 μl of HCl at various concentrations for 5 min at room temperature and then serially diluted in 1 ml of phosphate-buffered saline (PBS). One-hundred-microliter aliquots of each dilution were then plated onto heart infusion agar. After the 5-day microaerobic incubation, colonies were counted. Control cultures were carried out using PBS instead of urea-HCl under the same conditions. Additional experiments were conducted to assess incubation time and temperature (4 to 37°C) effects on the survival of *H. pylori* exposed to urea-HCl. Second, saliva spiked with different concentrations of *H. pylori* was tested using the procedures above. Optimal urea and HCl concentrations were determined based on *H. pylori* survival and minimal growth of other microorganisms. Third, the minimum number of *H. pylori* CFU that could be inoculated into saliva and successfully recovered was determined using the optimal urea-HCl treatment conditions as determined from experiments described above. One milliliter of saliva spiked with 10 to 10^4 CFU of *H. pylori* was evaluated. Centrifugation was used instead of the PBS dilution to remove urea-HCl after the pretreatment. Urease activities of viable intact cells of the type strain and clinical isolates were measured using a coupled enzyme assay (5, 14).

Exposure of pure *H. pylori* to HCl-urea mixtures resulted in survival rates of 0.01 to 70% in a pH range from 0.36 to 2.7. In the absence of urea, few *H. pylori* organisms survived. Incubation for 1 and 20 min in 0.06 N HCl–0.08 M urea (pH 1.2) gave similar *H. pylori* recoveries (range, 6 to 10%), but ≥1 h killed nearly all *H. pylori* cells. Incubation in 0.06 N HCl–0.08 M urea at 37, 25, and 4°C for 5 min gave 15.7, 10.4, and 0.8% survival rates, respectively ($P < 0.001$).

Hydrochloric acid concentrations of 0.06 N or higher were necessary to effectively inhibit microflora present in saliva. Table 1 shows survival rates of *H. pylori* added to saliva after pretreatment using urea-HCl for 5 min at room temperature. Optimal HCl-urea concentrations were 0.06 N HCl–0.02 to 0.16 M urea, 0.12 N HCl–0.2 to 0.5 M urea, and 0.24 N HCl–1 M urea, with all giving nearly equivalent recoveries of *H. pylori* (Table 1). The 0.06 N HCl–0.08 M urea combination (arbitrarily selected) and 5-min incubations at 37°C were used in the further experiments. *H. pylori* was consistently and readily isolated at inoculum levels as low as 10^2 CFU/ml of saliva. In contrast, a minimum concentration of 10^4 CFU of *H. pylori*/ml in saliva was necessary for successful isolation on Skirrow's medium without urea-HCl pretreatment.

Twenty-five gastric biopsy specimens were subjected to *H. pylori* isolation using the optimal urea-HCl pretreatment method described above. The results were compared with those obtained by direct inoculation onto Skirrow's medium.

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TABLE 1. Survival rate of *H. pylori* strain ATCC 43504^T added to saliva after a short exposure to urea and HCl

HCl concn (N)[b]	Survival (%) at[a]: Urea concentration (mM)[b]										pH
	1,000	500	200	160	80	40	20	10	5	2.5	1.25	
0.24	4.75	3.2	1.6	1.15	0.8	0.48	0.48	0.21	0.48	0.32	0.16	0.68
0.12	2.5	5.5	4.75	3.8	2.75	1.25	2.05	0.55	0.23	0.8	0.03	1.06
0.06	2.15	3.45	3.73	4.5	4.2	3.9	4.75	1.45	0.9	1.75	0.02	1.38
0.03	0.65	1	1.25	1	1.95	1.5	2.6	0.8	0.34	0.38	0.01	1.82

[a] *H. pylori* survival rates are expressed as the percentage of input colonies recovered after urea-HCl treatment, as determined by parallel controls with PBS replacing urea and HCl. All values are means for triplicate trials.
[b] Final concentration during incubation of *H. pylori* suspension with urea and HCl solution before dilution with PBS buffer.

Similar isolation rates were obtained: 56% (14 of 25) for urea-HCl pretreatment and 52% (13 of 25) for Skirrow's medium ($P > 0.05$). With one specimen, *H. pylori* colonies were isolated only after urea-HCl pretreatment; direct plating of this specimen in Skirrow's medium resulted in overgrowth by competitors. After exposure to the optimal conditions, 13 clinical strains had survival rates of 14 to 86% (median, 49%), compared with 8% for the type strain ($P < 0.01$). Urease activities ranged from 1.4×10^{-8} to 4.6×10^{-8} (median, 2.5×10^{-8}) μM ammonia/min/cell for 13 clinical strains and 1.3×10^{-8} μM ammonia/min/cell for the type strain ($P < 0.05$). While the survival rate of *H. pylori* appeared to increase with increasing urease activity, this was not a statistically significant association ($P > 0.10$).

Our data show that in the presence of appropriate concentrations of urea, *H. pylori* can survive short periods of exposure to acid at much lower pH levels than previously reported (3, 11, 12, 15). The conditions used in our study are similar to those occurring in natural *H. pylori* infection, where *H. pylori* and other organisms enter the stomach through the mouth (4, 23). Before reaching the gastric mucosa, *H. pylori* encounters the acidic (pH 1 to 6) stomach contents. It is postulated that a large proportion of *H. pylori* cells are killed during the stomach exposure, with a smaller number surviving.

Our finding is similar to those of other reports in that *H. pylori* could not be isolated from saliva using standard culture conditions, particularly when the number of *H. pylori* organisms in the sample was lower than 10^4/ml, because of overgrowth by other oral organisms (13). Other investigations have demonstrated that the *H. pylori* load in the oral cavity is quite low (20). It is highly likely that the number of *H. pylori* in the natural environment, water, and other potential infection sources is also very low because of the fastidious nature of the organism. Therefore, applying the method described here may yield better success in culturing *H. pylori* from extragastric contaminated sites. This approach may be particularly useful for epidemiologic studies to identify the source and route of transmitting *H. pylori*.

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