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Optimizing Enrichment Culture Conditions for Detecting *Helicobacter pylori* in Foods

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ABSTRACT

The survival and growth of *Helicobacter pylori* under enrichment conditions in fresh, autoclaved and irradiated ground beef were determined. *H. pylori* grew in autoclaved ground beef at 37°C under microaerobic conditions in brain heart infusion broth with 7% horse serum at pH 7.3 after 3 to 7 days of lag time but did not grow within 7 days in irradiated (10 kGy) ground beef under the same enrichment conditions. Adjustment of the enrichment broth to pH 5.5 enabled the growth (ca. 2 $\log_{10}$ CFU/ml) of *H. pylori* within 7 days in the presence of irradiated ground beef and the prolific growth (ca. 3 to 4 $\log_{10}$ CFU/ml) of *H. pylori* within 3 days in the presence of autoclaved beef. *H. pylori* in fresh ground beef could not be isolated from enrichment media with antibiotics; however, *H. pylori ure*A could be detected by polymerase chain reaction (PCR) in such enrichment media after 1 to 3 days of incubation at 37°C. The addition of supplements, i.e., 0.3% mucin, 0.05% ferrous sulfate, and 0.05% sodium pyruvate or 0.008 M urea, or the adjustment of the enrichment broth pH to 5.5 or 4.5 enabled the detection of *H. pylori ure*A in enrichment media incubated for 1, 2, 3, and/or 7 days at 37°C. *H. pylori* in sterile milk refrigerated at 4°C at an initial level of 10^6 CFU/ml was inactivated to an undetectable level within 6 days; however, *H. pylori* was not detected either by a PCR assay or by the plating of enrichment cultures of 120 raw bovine milk samples.

Helicobacter pylori is the principal cause of gastric ulcers in humans; this organism is able to colonize the stomach, in which most other bacteria cannot tolerate the high acidity. This pathogen also causes stomach cancer and has been designated a type I carcinogen by the World Health Organization *(4)*.

The routes by which *H. pylori* is transmitted to humans have not been fully elucidated, but both waterborne and foodborne pathways have been suggested *(3, 5, 7, 11)*. By using a monoclonal antibody against *H. pylori,* Hegarty et al. *(5)* detected *H. pylori* in most of the surface and shallow ground water samples they tested in Pennsylvania and Ohio. The DNA of *Helicobacter* was also detected by a polymerase chain reaction (PCR) method in a biofilm present in a water distribution system in northeast Scotland *(7)*. Dore et al. *(3)* used a traditional culture method to recover *H. pylori* from raw ovine milk and suggested that *H. pylori* may be a commensal in sheep. Dimola and Caruso *(2)* isolated *H. pylori* from large animals, such as calves, pigs, and horses, but not from rabbits or chickens. However, Stevenson et al. *(9)* failed to isolate *H. pylori* from either cattle or beef products.

Many researchers have reported difficulty in detecting *H. pylori* in environmental samples due to the pathogen's fastidious and microaerobic growth requirements and the large numbers of competitive bacteria in such samples *(9, 10, 12)*. Hence, the successful detection of this pathogen in contaminated food and environmental samples will depend on enrichment culture conditions that stimulate the growth of *H. pylori* and inhibit the growth of indigenous contaminating bacteria. In a previous study, we identified some growth supplements, such as mucin, ferrous sulfate, and sodium pyruvate, that enhance the growth of *H. pylori* in a nutrient medium in the presence of five highly selective antibiotics *(6)*. In this study, our objectives were to design an enrichment medium with growth supplements and selective agents that enable the growth and detection of *H. pylori* in foods such as ground beef, to determine the fate of *H. pylori* in food during refrigerated storage, and to use our enrichment medium to assay raw bovine milk samples for the presence of *H. pylori*.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *H. pylori* strains NB2-1 (human isolate), G2-1 (human isolate), 26695 (human isolate), WV99 (human isolate), and 1324P-1 (monkey isolate) were obtained from Dr. Douglas Berg (Washington University, St. Louis, Mo.). A green fluorescent protein-labeled *H. pylori* P1 pDH80 strain was kindly provided by Dr. R. Haas (Max von Pettenkofer Institut für Hygiene und Medizinische Mikrobiologie, Munich, Germany). Bacteria were grown on plates of brain heart infusion agar supplemented with 7% horse serum (BHI-HS). Cultures were incubated at 37°C for 3 days in a GasPak jar (BBL Microbiology Systems, Cockeysville, Md.), which was evacuated three times and replaced with a microaerobic gas mixture comprising 5% oxygen, 10% carbon dioxide, and 85% nitrogen.

Microbiological analysis of fresh ground beef. Fresh ground beef was purchased from a local grocery store. The meat samples were plated immediately on BHI-HS and incubated at

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37°C both aerobically and microaerobically for 3 to 5 days. Colonies with different morphologies were picked and streaked onto BHI-HS for purity. The isolates were tested for resistance to antibiotics, including trimethoprim lactate, amphotericin B, cefsulodin, polymyxin B, and vancomycin, and those isolates found to be resistant to antibiotics were identified with API identification kits (bioMerieux Vitek, Hazelwood, Mo.).

Irradiation treatment of ground beef. Fresh ground beef (80% lean) purchased at a supermarket was placed in sterile stomacher bags (250-g portions) and heat sealed. The meat was frozen overnight and then irradiated at the Neely Nuclear Research Center, Georgia Institute of Technology, Atlanta, Ga. The frozen samples were tied with binder clips to a string crossing the irradiation chamber and arranged in a row to receive the same dose (10 kGy) of irradiation from a Cobalt-60 source (with gamma energies of 1.173 MeV and 1.331 MeV) at a rate of 5 kGy/h. The dose absorbed by ground beef samples was measured with a Keithley autoranging picoammeter model 485 with an LND ionization chamber probe (Keithley Instruments, Inc., Cleveland, Ohio).

Inoculation of food with *H. pylori*. The five strains of *H. pylori* were grown individually on BHI-HS for 3 days at 37°C under microaerobic conditions. Each culture was scraped from its agar plate and suspended in 0.01 M phosphate-buffered saline (PBS, pH 7.2). Cells were sedimented by centrifugation at 1,500 $\times$ *g* for 10 min, and the pellets were resuspended in PBS to an optical density of 0.5 (ca. 5×10^8 CFU/ml) at 630 nm. *H. pylori* populations for each individual strain and for the five-strain mixture were enumerated on BHI-HS. *H. pylori* inoculum (2.5 ml) was added aseptically under a laminar flow hood to 247.5 g of ground beef in a sterile stomacher bag for a final population of ca. 5×10^5 CFU of *H. pylori* per g. The inoculated ground beef was then pummeled at medium speed in a Stomacher 400 Laboratory Blender (Teckman, Cincinnati, Ohio) for 2 min for uniform distribution. Pasteurized apple and orange juices (purchased from a supermarket) and sterile skim milk were inoculated with *H. pylori* at a ratio of 1:100. The inoculated food samples were then held at 4°C under microaerobic conditions.

Uninoculated food samples were assayed for pH, aerobic plate counts, and *H. pylori* counts. Aerobic plate counts for foods were determined by plating serial dilutions (1:10) in 0.01 M PBS (pH 7.2) on plate count agar and incubating them at 35°C for 48 h. The populations of *H. pylori* in inoculated food samples were determined at selected intervals during storage at 4°C. Food samples (25 g for solid samples and 1 ml for liquid sample) were diluted (1:10) in 0.01 M PBS in a stomacher bag. The solid food samples were pummeled at medium speed in a Stomacher 400 Laboratory Blender for 2 min. As for pasteurized juice samples, a neutralizing buffer (Difco) was used for serial dilution. Serial dilutions were prepared and surface plated in duplicate on nonselective BHI-HS for sterile food samples and on selective BHI-HS agar containing an *H. pylori*–selective supplement (Dent supplement) comprising trimethoprim lactate (5 mg/liter), vancomycin (10 mg/liter), amphotericin B (5 mg/liter), cefsulodin (5 mg/liter; Oxoid, Hampshire, UK), and filter-sterilized (0.2 μm Nalgene syringe filter) polymyxin B solution (2,500 IU/liter) for fresh ground beef samples. The media were incubated under microaerobic conditions for 3 to 7 days at 37°C. Randomly selected colonies (an average of five per plate) from media with the highest dilution were confirmed as *H. pylori* by microscopic examination, by urea tests, and with catalase, oxidase, and API-CAMPY biochemical identification kits (bioMerieux Vitek, Hazelwood, Mo.). When *H. pylori* was not detectable by direct plating, samples were assayed by enrichment culture. BHI-HS broth was used as the base medium to which different combinations of growth supplements were added, and the pH was adjusted. Porcine stomach mucin (0.3%; Sigma Chemical Co., St. Louis, Mo.) was added prior to the autoclaving of the enrichment broth, whereas ferrous sulfate and sodium pyruvate (FP, 5%) or urea (0.8 M; Sigma Chemical Co.) were filter sterilized (0.2 μm Nalgene syringe filter) and added to the enrichment broth at a ratio of 1:100 after heat treatment. The pH of sterile enrichment broth was adjusted to either 5.5 or 4.5 with sterile 0.1 N HCl. The samples were incubated in the enrichment broth at 37°C for 72 h with agitation (150 rpm) under microaerobic conditions.

PCR detection of *H. pylori* in fresh ground beef. DNA from *H. pylori* was extracted with an InstaGene Matrix kit (BioRad). Two oligonucleotide primers, designated HP1 (5′-GCCAATGGTAAATTAGTT-3′) and HP2 (5′-CTCCTTAATTGTTTTTAC-3′), were derived from *H. pylori ure*A *(1)*. This primer set amplifies a 411-bp DNA fragment of the *ure*A gene. PCR buffer (80 μl) containing 0.4 μM HP1 and HP2 primers and 2.5 U of AmpliTaq polymerase was added. The reaction mixtures were heated at 94°C for 5 min for DNA denaturation and were then amplified with 33 cycles consisting of 1 min of denaturation at 94°C, 1 min of annealing at 48°C, and 1 min of primer extension at 72°C. After the 33rd cycle, the extension step was continued for 5 min at 72°C. Amplified DNA fragments were resolved by gel electrophoresis using 2% (wt/vol) agarose. Gels were stained with 0.5 μg of ethidium bromide per ml, visualized with UV illumination, and imaged with a Polaroid instant camera system.

Testing raw bovine milk for *H. pylori*. One hundred twenty raw samples of bovine milk were collected from a major dairy processing plant at Lawrenceville, Ga., over a 7-month period (from August 2000 to February 2001). Most samples were collected within 12 to 48 h after delivery from the farms. The samples were brought to the laboratory on ice and analyzed immediately on arrival. The enrichment broth consisted of BHI-HS broth (pH 5.5) with 0.3% mucin and antibiotics (5 mg of amphotericin B per liter, 10 mg of vancomycin per liter, 5 mg of trimethoprim per liter, 5 mg of cefsulodin per liter, and 2,500 IU of polymyxin B per liter). The milk samples in the enrichment broth were incubated at 37°C with agitation (150 rpm) under microaerobic conditions. After 3 days of enrichment, milk samples were both streaked onto selective BHI-HS agar and analyzed for *ure*A by the PCR assay. Colonies typical of *Helicobacter* were further confirmed by the urease enzyme test and by microscopic observation.

RESULTS

Survival of *H. pylori* in ground beef, sterile skim milk, and pasteurized fruit juices. *H. pylori* survived at 4°C in fresh ground beef for up to 7 days in one of two samples (Fig. 1A). On day 14, the detection of *H. pylori* was hampered by a high level of background bacteria, i.e., an aerobic plate count of $>10^7$ CFU/g. In ground beef irradiated at 10 kGy and in sterile skim milk, the population of *H. pylori* decreased from 10^4 to 10^5 CFU/g or from 10^4 to 10^5 CFU/ml of food to an undetectable level (<10 CFU/g or <10 CFU/ml) within 11 and 6 days, respectively (Fig. 1B and 1C). Approximately the same *H. pylori* counts were obtained on BHI-HS agar with and without selective antibiotics (Fig. 1B and 1C). *H. pylori* P1 pDH80, with an initial population of 10^4 CFU/ml in the pasteurized apple

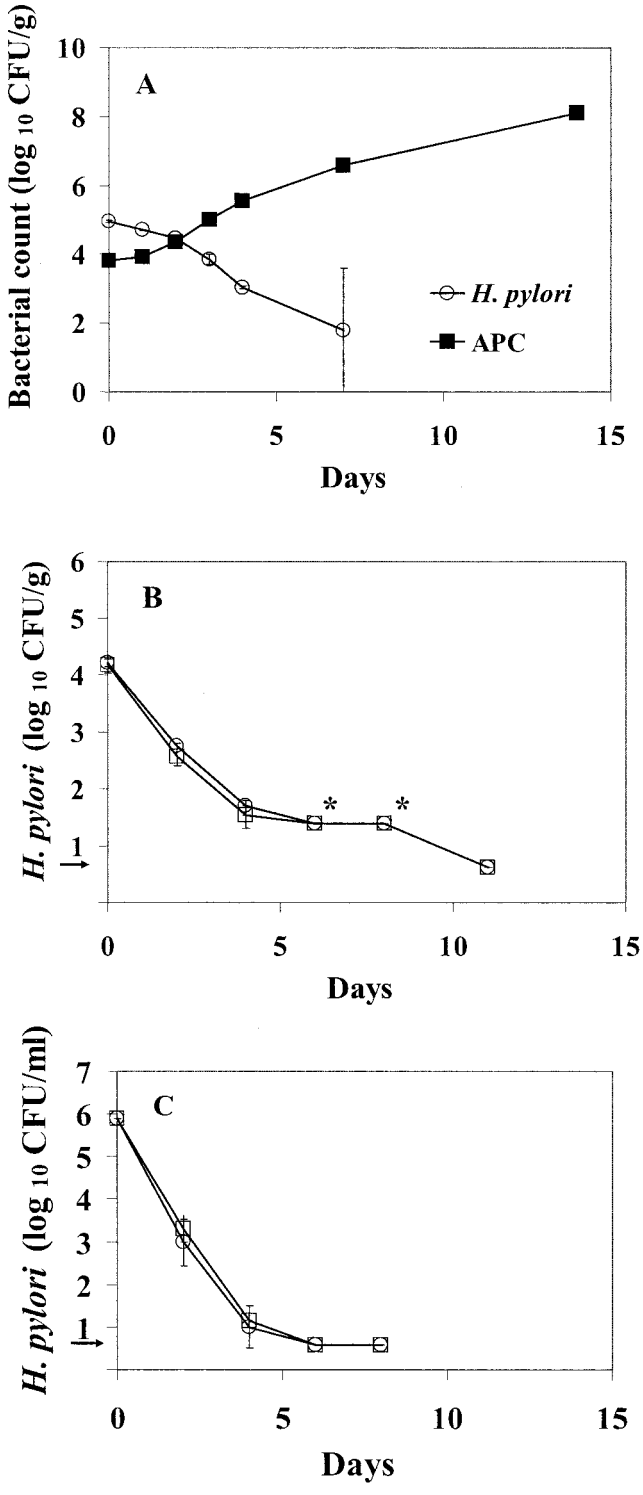

FIGURE 1. *Survival of a five-strain mixture of* H. pylori *in (A) fresh ground beef, (B) ground beef irradiated at 10 kGy, and (C) sterile skim milk as enumerated on selective BHI-HS plates (○) and on BHI-HS plates (□) during storage at 4°C.* ■, *aerobic plate counts in fresh ground beef. Each datum point represents an average of duplicate results. An asterisk indicates that* H. pylori *was detected by enrichment only, whereas an arrow indicates that the level of* H. pylori *was below the detection limit (<10 CFU/g or CFU/ml) and the organism was not detected by enrichment.*

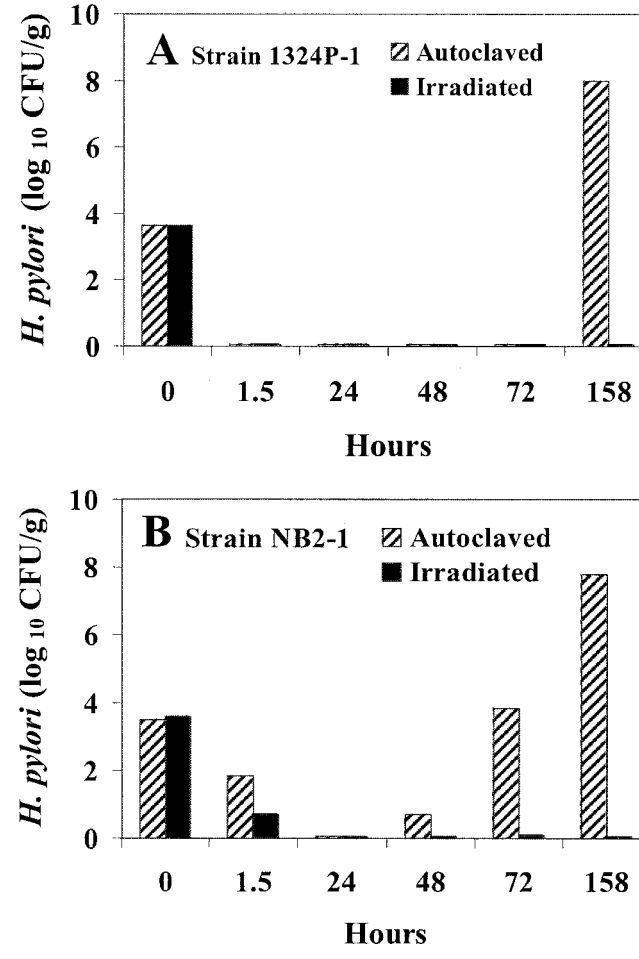

FIGURE 2. *Growth of* H. pylori *strains 1324P-1 (A) and NB2-1 (B) in BHI-HS enrichment broth (pH 7.3) at 37°C with autoclaved (///) and 10 kGy–irradiated (solid bar) ground beef. Each datum point represents an average of duplicate results.*

and orange juices, was inactivated rapidly to an undetectable level (<10 CFU/ml) within 24 h when held at either 4 or 21°C (data not shown). The pH values for both juices were of approximately 3.8.

Enrichment of *H. pylori* in artificially inoculated sterile ground beef. Two *H. pylori* strains, the slowly growing strain 1324P-1 and the more rapidly growing strain NB2-1, were used to determine the best enrichment protocol for isolating *H. pylori* from ground beef. At 37°C with agitation under microaerobic conditions, *H. pylori* multiplied in BHI-HS enrichment broth (pH 7.3) with autoclaved ground beef, but only after 3 and 7 days of lag time for strains NB2-1 and 1324P-1, respectively (Fig. 2A and 2B). In contrast, neither *H. pylori* strain grew in the enrichment broth with irradiated ground beef during 7 days of incubation. Interestingly, there were 3.6- and 2.9-log reductions in culturable cells of strains 1324P-1 and NB2-1, respectively, when these strains were held in irradiated ground beef for 1.5 h at 25°C (Fig. 2A and 2B). Strain 1324P-1 populations decreased more rapidly during the initial 1.5 h of incubation in both autoclaved and irradiated ground beef than did strain NB2-1.

The effects of growth supplements (such as mucin), FP,

and the adjustment of the pH of the enrichment broth were evaluated to determine the best enrichment conditions for *H. pylori.* In autoclaved ground beef, *H. pylori* grew best in enrichment broth supplemented with 0.3% mucin at pH 5.5 (Fig. 3A). Within 3 days of incubation, *H. pylori* populations had increased by approximately 5 log CFU/ml in enrichment broth with mucin supplement. Enrichment broth (pH 5.5) supplemented with FP or with mucin plus FP was as effective as unsupplemented enrichment broth (pH 5.5) in supporting the growth of *H. pylori.* In irradiated ground beef, the growth of strain NB2-1 in enrichment medium at pH 5.5 was enhanced by the addition of 0.3% mucin but not by the addition of FP or a combination of mucin and FP (Fig. 3B). Even without mucin and FP supplements, *H. pylori* in irradiated ground beef grew when the enrichment broth was adjusted to pH 5.5. However, without adjustment of the pH of the enrichment broth (pH 7.3), *H. pylori* did not grow within 7 days of incubation in enrichment broth with irradiated ground beef whether or not supplements were used (Fig. 3C).

Enrichment culture of *H. pylori* in inoculated fresh ground beef. Seventy-five bacterial isolates from three batches of ground beef (80% lean, 20% fat) were selected and tested for sensitivity to trimethoprim and vancomycin. Twenty-six isolates (35%) were found to be resistant to both antibiotics. These isolates included fluorescent *Pseudomonas* spp., *Acinetobacter* spp., *Serratia liquefaciens,* and *Aeromonas hydrophilia.* On the basis of these results, a combination of five antibiotics, trimethoprim, vancomycin, amphotericin, cefsulodin, and polymyxin B, were selected as selective agents for use in the enrichment broth used for the growth of *H. pylori* in fresh ground beef.

Autoclaved ground beef and sterile skim milk were first tested to evaluate the selective agents in the enrichment broth used for culturing *H. pylori.* A five-strain mixture of *H. pylori* was inoculated into the sterile food at an initial inoculum of 2×10^4 CFU/g or 2×10^4 CFU/ml and incubated in the selective enrichment broth at 37°C under microaerobic conditions. After 4 days of enrichment incubation, *H. pylori* growth was detected in all enrichment cultures. Selective enrichment broth supplemented with 0.3% mucin and 0.05% FP was slightly more effective than selective BHI-HS broth for the growth of *H. pylori* (data not shown). The detection limit of *H. pylori* in sterile skim milk was as low as 10 CFU/ml.

The fresh ground beef contained approximately 4.0×10^4 CFU of microaerobic bacteria per g, and approximately 1% of these bacteria were resistant to the five antibiotics used in this selective medium. *H. pylori* NB2-1 was inoculated into fresh ground beef at 2.0×10^4 CFU/g. Even when 0.3% mucin or 0.3% mucin plus 0.05% FP was added to the selective enrichment broth and the pH was adjusted to either 5.5 or 4.5, *H. pylori* was not recovered from any fresh ground beef samples after 24 h of enrichment culture.

The PCR assay detected *H. pylori ure*A DNA for 1 day in unsupplemented enrichment medium with fresh ground beef, for 2 days in supplemented enrichment medium (pH 7.3) with fresh ground beef, and for 7 days in supplemented enrichment medium (pH 5.5 or 4.5) with fresh ground beef (Table 1). Dilution of the 24-h supplemented enrichment media (both pH 7.3 and 5.5) by 1:10

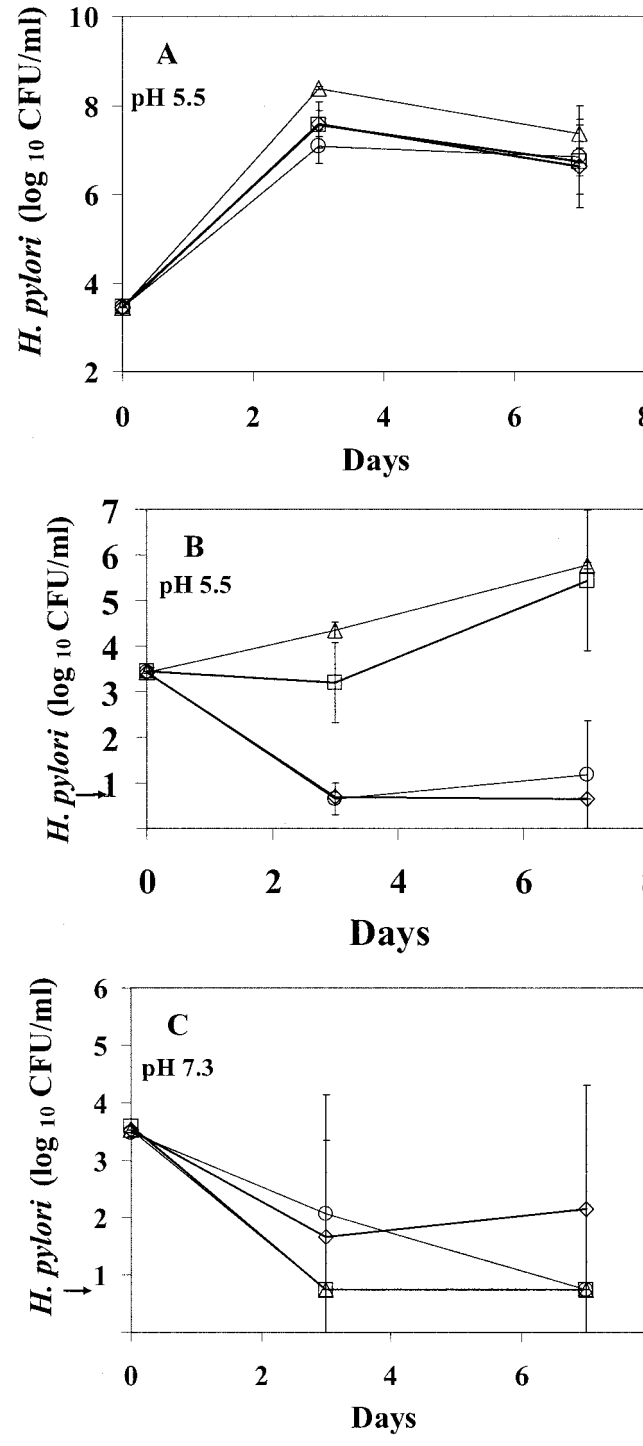

FIGURE 3. *Growth of* H. pylori *NB2-1 at 37°C in (A) enrichment broth with autoclaved ground beef (pH 5.5), (B) enrichment broth with irradiated ground beef (pH 5.5), and (C) enrichment broth with irradiated ground beef (pH 7.3). Symbols indicate BHI-HS enrichment broth without supplements (□), with 0.3% mucin (△), with 0.05% FP (◇), and with 0.3% mucin and 0.05% FP (○). Each datum point represents an average of duplicate results. An arrow indicates that the level of* H. pylori *was below the detection limit (<10 CFU/ml).*

TABLE 1. *PCR detection of* H. pylori *NB2-1 in enrichment broth with* H. pylori*–inoculated ground beef*

Enrichment[a]	Detection after storage time at 37°C[b]				
	0 days	1 day	2 days	3 days	7 days
Autoclaved ground beef	+	+	+	+	+
Fresh ground beef					
Without supplements (pH 7.3)	+	+	−	−	−
With supplements (pH 7.3)	+	+	+	+/−	−
With supplements (pH 5.5)	+	+	+	+	+
With supplements (pH 4.5)/urea	ND	+	+	+	+
With supplements (pH 4.5)	ND	ND	+	+	+
With supplements (pH 7.3), 1:10	ND	ND	−	−	+
With supplements (pH 5.5), 1:10	ND	ND	−	−	+

[a] Enrichment broth consisted of 90 ml of BHI-HS with a combination of five antibiotics. The pH of the enrichment broth was adjusted to 5.5 or 4.5 with sterile 0.1 N HCl. Supplements included 0.3% mucin, 0.05% ferrous sulfate, and 0.05% sodium pyruvate. 1:10, 10 ml of original enrichment broth was added to 90 ml of fresh enrichment broth after 24 h of incubation.

[b] +/−, detected in one but not both samples; ND, not detected.

enabled the detection of *H. pylori ure*A DNA on day 7 of enrichment culture but not on day 2 or 3.

Assay of raw bovine milk for *H. pylori*. One hundred twenty raw bovine milk samples were assayed for *H. pylori* both by direct plating onto selective BHI-HS and by PCR assay of enrichment culture. *H. pylori* was not detected in any of the samples.

DISCUSSION

H. pylori is a microaerobic and fastidious gram-negative bacterium. It does not grow well in an environment that contains large populations of competitive microorganisms; hence, sterile food was used in initial studies to evaluate the survival and growth of *H. pylori* in enrichment media. *H. pylori* grew well in autoclaved ground beef in BHI-HS broth during enrichment incubation. However, the growth of *H. pylori* in enrichment medium at pH 7.3 with irradiated ground beef was markedly affected, with substantially fewer helicobacters in enrichment medium with irradiated than with autoclaved ground beef held at 37°C for as little as 1.5 h (Fig. 2B). When free radical quenchers such as Tempol (1 mM), caffeine (1 mM), carosine (5 mM), and catalase (100 U/ml) were added to the enrichment broth with irradiated ground beef, there was no improvement in the recovery time for or the growth of *H. pylori* during enrichment culture (data not shown). These results suggest that *H. pylori* is very sensitive to chemical changes that occur in irradiated ground beef but not to those that occur in autoclaved ground beef. Reduction of the pH of the enrichment broth from 7.3 to 5.5 greatly enhanced the survival, growth, and detectability of *H. pylori* in foods and should be considered an important factor in the detection of *H. pylori* in enrichment cultures.

The microfloras of foods are quite different from those of fecal or clinical origin. Therefore, selective isolation media developed for use with clinical specimens may not successfully isolate pathogens from food samples. In addition, the number of *H. pylori* cells in clinical specimens is likely considerably larger than that in contaminated food or environmental samples. Dent supplement, which contains four antibiotics, is commercially available and is employed in media used for the isolation of *H. pylori* from clinical specimens. In this study, in addition to Dent supplement, polymyxin B was included because of its ability to suppress the large number of *Pseudomonas* spp. present in fresh ground beef. Approximately 10^4 *H. pylori* cells were inoculated into 1 g of food in this study. The five selective agents used in isolation media suppressed the growth of >99% of the indigenous bacteria in fresh ground beef initially; however, after 24 h of enrichment culture, those small numbers of indigenous bacteria outgrew *H. pylori*. We previously determined that the addition of mucin and FP to BHI-HS broth with antibiotics counteracted the inhibitory activity of the five antibiotics against *H. pylori (6)*. In this study, the pathogen grew well in the antibiotic-supplemented selective enrichment broth with autoclaved food. However, in fresh ground beef, small numbers of *H. pylori* could not compete with a small number of resistant indigenous bacteria during enrichment incubation. An enrichment medium containing a combination of antibiotics including vancomycin, amphotericin B, cefsulodin, polymyxin B, and sulfamethoxazole was recently investigated by Stevenson et al. *(9)*. This medium suppressed the growth of *H. pylori* during the first 24 h but suppressed growth only slightly at 48 h. However, studies are needed to determine the efficacy of the enrichment broth in isolating *H. pylori* from foods, especially foods with high populations of indigenous microorganisms.

Our study revealed that a PCR assay based on *ure*A is useful for detecting *H. pylori* in enrichment cultures of *H. pylori*–contaminated foods. The addition of mucin, FP, or urea to the enrichment broth, along with adjustment of the pH to 5.5 or 4.5, enhanced the survival and possibly enabled the growth of *H. pylori* in enrichment medium with fresh ground beef.

H. pylori was detected in one of 51 raw ovine milk samples obtained from northern Sardinia by using a direct plating method *(3)*. In our study, 120 raw bovine milk samples from various farms were tested, but *H. pylori* was not

detected in any samples. Mucosal samples collected from the rumen and abomasum of 105 cattle, as well as 20 retail beef cuts assayed for *H. pylori* by direct plating and by enrichment culture, all tested negative for the pathogen *(8)*. These results indicate either that the likelihood of transmission of *H. pylori* by cattle or beef products is very low or that more sensitive methods for the detection of *H. pylori* in tissue or food specimens need to be developed.

The results of this study indicate that *H. pylori* can survive for a few days in foods during refrigerated storage but does not grow prolifically in foods even in enrichment culture media. The PCR assay appears to be more successful than a plating method for the detection of *H. pylori* in enrichment media; however, a positive PCR assay does not ensure that the pathogen is viable in food.

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