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Development of a Selective Medium for Isolation of *Helicobacter pylori* from Cattle and Beef Samples

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Helicobacter pylori **has been isolated from the human stomach with media containing only minimal selective agents. However, current research on the transmission and sources of infection requires more selective media due to the higher numbers of contaminants in environmental, oral, and fecal samples. The objective of this study was to develop and evaluate detection techniques that are sufficiently selective to isolate *H. pylori* from potential animal and food sources. Since *H. pylori* survives in the acidic environment of the stomach, low pH with added urea was studied as a potential selective combination. *H. pylori* grew fairly well on *H. pylori* Special Peptone plating medium supplemented with 10 mM urea at pH 4.5, but this pH did not sufficiently inhibit the growth of contaminants. Various antibiotic combinations were then compared, and a combination consisting of 10 mg of vancomycin per liter, 5 mg of amphotericin B per liter, 10 mg of cefsulodin per liter, 62,000 IU of polymyxin B sulfate per liter, 40 mg of trimethoprim per liter, and 20 mg of sulfamethoxazole per liter proved to be highly selective but still allowed robust colonies of *H. pylori* to grow. This medium was highly selective for recovering *H. pylori* from cattle and beef samples, and it is possible that it could be used to enhance the recovery of this bacterium from human and environmental samples, which may be contaminated with large numbers of competing microorganisms.**

In 1982 Warren and Marshall (17) isolated a new spiral-shaped bacterium from the stomachs of humans with gastric ulcers, which was named *Campylobacter pyloridis*. Further research on this novel bacterium revealed significant morphological, biochemical, and genetic differences from other *Campylobacter* spp., and the microorganism was eventually renamed *Helicobacter pylori* (7). Since then, it has been determined that *H. pylori* is the most prevalent bacterial pathogen in the world and infects approximately one-half of the world's population (10). Infection with *H. pylori* causes numerous medical conditions, including gastritis, peptic ulcers, gastric carcinomas, and mucosa associated lymphoid tissue tumors (2, 9, 12).

It has not been necessary for the media used to isolate *H. pylori* from the stomach to be highly selective because relatively few contaminants survive in the low-pH environment of the stomach (13). However, current research on the transmission and sources of infection requires more selective media. In several studies workers have tried to determine the prevalence of *H. pylori* in the oral cavities or feces of infected patients, but these efforts have been hindered by the large number of contaminants and the fastidious nature of the bacterium (3, 5, 16). Therefore, the need for a more selective medium has become increasingly important.

The objective of this study was to develop and evaluate detection techniques that are selective enough so that *H. pylori* can be isolated from potential animal and food sources. Since *H. pylori* has the unique ability to survive in the highly acidic environment of the stomach, techniques that capitalize on this distinctive feature were examined. Lee (11) proposed that the spiral morphology and rapid motility of *H. pylori* allow the bacterium to penetrate the viscous coating of the stomach and attach to the gastric mucosa and thus escape the harsh acidic environment in the lumen of the stomach.

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Another distinguishing feature of *H. pylori* is its exceptionally high urease activity, which purportedly helps the bacterium raise the pH of the surrounding fluid to a level which it can tolerate (6). In addition to the possible protective effects of urease in the acidic gastric environment, Williams et al. (18) found that *H. pylori* utilizes urea as a nitrogen source for amino acid synthesis. We surmised that urea might be added to a selective medium at a low pH to further enhance the survival of the organism. We also examined the effectiveness of additional selective agents, such as antibiotics and inhibitory dyes that are routinely used to isolate enteric pathogens.

MATERIALS AND METHODS

Source and maintenance of cultures. Isolates of *H. pylori* (strains ATCC 43504, ATCC 43629, and ATCC 43579, all of which originated from human gastric samples) were obtained from the American Type Culture Collection, Rockville, Md. These organisms were maintained on *H. pylori* Special Peptone agar (HPSPA) plates and were streaked onto fresh plates every 3 or 4 days (14a). The plates were incubated at 37°C in 5.5-liter boxes (AnaeroPack; Mitsubishi Gas Chemical Co., New York, N.Y.) that were flushed with a microaerophilic gas mixture (6% O_2, 10% CO_2, 84% N_2), which was used throughout this study.

Standard plating medium. Comparative studies performed in our laboratory confirmed that the rate of growth and the subsequent colony size of *H. pylori* were significantly greater on HPSPA than on any of the other media routinely used to culture the bacterium (14a). HPSPA was prepared by adding 10 g of Special Peptone (Oxoid Ltd., Basingstoke, England) per liter, 15 g of granulated agar (Difco Laboratories, Detroit, Mich.) per liter, 5 g of sodium chloride (EM Science, Gibbstown, N.J.) per liter, 5 g of yeast extract (Difco) per liter, 5 g of beef extract (Becton Dickinson and Co., Cockeysville, Md.) per liter, and 0.5 g of pyruvic acid (sodium salt; Sigma Chemical Co., St. Louis, Mo.) per liter to water.

Growth on acidified agar. *H. pylori* Special Peptone broth (HPSPB), brain heart infusion broth, and Mueller-Hinton broth were inoculated with *H. pylori* ATCC 43504 in order to prepare inocula that were plated onto acidified HPSPA (pH 7.0, 6.5, 6.0, 5.5, and 5.0). Acidified agar was prepared by adding HCl to the autoclaved media and measuring the pH to ±0.1 pH unit with a pH meter (model 612; Markson Science, Inc., Phoenix, Ariz.). Broth media were inoculated with cells that had been harvested from one HPSPA plate with a sterile cotton swab, dispersed in 9 ml of 0.1% peptone water (Difco), and vortexed for 10 s. The same volume of the peptone water inoculum was dispensed into each 250-ml Erlenmeyer flask (five flasks per medium) containing 96 ml of room temperature broth and 4 ml of iron-supplemented calf serum (Biologos, Inc., Napersville, Ill.). Each flask was flushed with a microaerophilic gas mixture, sealed with a sterile rubber stopper, and incubated at 37°C. After the broth media were incubated for 24 and 48 h, samples were collected, and 0.1-ml portions of appropriate decimal

dilutions were surface plated onto HPSPA with different pH values; a total of 30 replicates were prepared for each plating medium. Following inoculation, all of the plates were placed in 5.5-liter plastic boxes (AnaeroPack) that were flushed with a microaerophilic gas mixture and incubated at 37°C for 4 days. After incubation, colonies were counted and measured by using a ruler and the magnifying lens of a Quebec colony counter (Leica, Buffalo, N.Y.). Typical *H. pylori* colonies (clear, circular, entire, convex, 0.5 to 1.5 mm in diameter) were identified on the basis of a positive urease reaction on Christensen's urea agar (4), a positive catalase test with hydrogen peroxide (Fisher, Fair Lawn, N.J.), and typical curved-rod morphology after primary staining with crystal violet (Difco).

Additional experiments were conducted in order to compare growth on HPSPA at lower pH values (pH 7.0, 5.5, 5.0, 4.5, 4.0, and 3.5). Cells were harvested from plates with a sterile cotton swab and dispersed in 9 ml of 0.1% peptone water. After the preparation was vortexed for 10 s, 0.1-ml portions of appropriate decimal dilutions were surface plated onto various plating media at different pH values. Following inoculation, all of the plates were incubated for 4 days as described above, and growth of *H. pylori* was evaluated by counting and measuring the colonies.

Growth on acidified agar containing urea. *H. pylori* was grown by using HPSPA and different combinations of pH and urea concentrations (pH 5.5 and no urea, pH 5.5 and 0.1 mM urea, pH 4.5 and no urea, pH 4.5 and 0.1 mM urea, pH 4.5 and 1 mM urea, pH 4.5 and 5 mM urea, pH 4.5 and 10 mM urea, pH 3.5 and no urea, pH 3.5 and 0.1 mM urea, pH 3.5 and 1 mM urea, pH 3.5 and 5 mM urea, pH 3.5 and 10 mM urea), and the results were compared. HPSPA batches having different pH values were prepared, and various amounts of urea (Sigma) were filter sterilized and added after the agar had been autoclaved and cooled to 48°C. Cells were grown in 250-ml Erlenmeyer flasks containing HPSPB (two flasks for each strain of *H. pylori* [ATCC 43504 and ATCC 43579]) in order to obtain inocula for the various plating media. After the broth preparations were incubated for 24 h, samples were collected from the broth preparations, and 0.1-ml portions of appropriate decimal dilutions were surface plated onto the various media. The plates were incubated for 4 days, and colonies were counted and measured as described above.

Growth in the presence of TTC. Fifteen flasks containing broth (five flasks containing HPSPB, five flasks containing Mueller-Hinton broth, and five flasks containing brain heart infusion broth) were inoculated as described above in order to obtain inocula for plating on standard HPSPA and HPSPA supplemented with 40 mg of triphenyl tetrazolium chloride (TTC) (U.S. Biochemical Corp., Cleveland, Ohio) per liter. After the broth preparations were incubated for 24 and 48 h, samples were collected from the broth preparations, and 0.1-ml portions of appropriate decimal dilutions were surface plated onto the various media; a total of 30 replicates were prepared for each plating medium. The plates were incubated for 4 days as described above, and growth of *H. pylori* was evaluated by counting and measuring the colonies.

Efficiency and selectivity of low pH values with various urea concentrations compared with antibiotics as selective agents. We compared the efficiency of pH 4.5 HPSPA containing added urea (10, 20, or 40 mM) with the efficiency of HPSPA containing four antibiotics (10 mg of vancomycin per liter, 10 mg of amphotericin B per liter, 10 mg of cefsulodin per liter, and 10 mg of trimethoprim per liter). Cells were grown in HPSPB (three flasks for each strain of *H. pylori* [ATCC 43504 and ATCC 43629]) in order to obtain inocula for the various plating media. After the broth preparations were incubated for 24 and 48 h, samples were collected from the broth preparations, and 0.1-ml portions of appropriate decimal dilutions were surface plated onto the various plating media; a total of 12 replicates were prepared for each plating medium. The plates were incubated for 4 days as described above, and growth of *H. pylori* was evaluated by counting and measuring the colonies.

We evaluated the selectivity of the media described above by collecting 9-cm^2 sections of mucosa from the rumen, the initial portion of the abomasum (omasoabomasal ostium), which corresponds to the cardia of the stomach of monogastric organisms, and the pyloric antrum of the abomasum of three cattle. These samples were ground for 30 s with 5 ml of 0.1% peptone water by using a mortar and pestle to release any *Helicobacter* spp. from the gastric pits within the mucosa, and 0.25-ml portions of the resulting fluid were then surface plated in duplicate onto standard HPSPA and selective HPSPA. After the noninoculated samples were plated, positive control plates were prepared by collecting three *H. pylori* colonies from an HPSPA plate with a sterile cotton swab and swirling the swab for 30 s in the fluid remaining in the mortar after the sample of mucosa had been ground with the pestle. All positive control samples were inoculated in the same manner. Portions (0.25 ml) of the inoculated sample fluid were then surface plated onto the various selective media. The plates were incubated as described above and examined after 4 and 7 days of incubation. Plates containing various selective agents were compared by evaluating the densities of contaminating colonies on the plates. Positive control plates were examined to determine whether they contained typical *H. pylori* colonies, which were identified as described above.

Selectivity of low pH compared with selectivity of four antibiotics when plates were inoculated with ground beef samples. Separate flasks containing HPSPB were inoculated with *H. pylori* ATCC 43504 or ATCC 43629 as described above and were incubated at 37°C for 48 h under microaerophilic conditions. Nine grams of fresh ground beef obtained from a local retail outlet was placed into a sterile stomacher bag and inoculated with 1 ml of broth containing ca. 6.4 $\log_{10}$ CFU of *H. pylori* per ml. Duplicate samples were inoculated for each strain of *H. pylori*, and a negative control was also included. Ninety milliliters of 0.1% peptone water (Difco) was added to the bag, and the sample was pummeled for 1 min with a model 400 stomacher (Tekmar Co., Cincinnati, Ohio). Then 0.1-ml portions of appropriate decimal dilutions were surface plated onto selective HPSPA plates. The plates were incubated for 4 days and then examined to determine whether they contained typical *H. pylori* colonies, which were counted with a Quebec colony counter (Leica) and identified as *H. pylori* colonies as described above.

Growth on HPSPA containing other combinations of antibiotics. Separate flasks containing HPSPB were inoculated with *H. pylori* ATCC 43504, ATCC 43629, or ATCC 43579 and incubated as described above. Samples were taken from the broth preparations after 24 and 48 h, and 0.1-ml portions of appropriate decimal dilutions were surface plated onto standard HPSPA and HPSPA supplemented with antibiotics. Various combinations of antibiotics were tested in nine separate trials. In all of the trials the plates were incubated for 4 days, and growth was evaluated by counting *H. pylori* colonies.

Selectivity of HPSPA containing additional antibiotics when it was inoculated with rumen and abomasum samples from cattle. HPSPA preparations containing various combinations of antibiotics were inoculated with samples obtained from the abomasum and rumen of six cattle as described above. The media contained a basic combination consisting of 10 mg of amphotericin B per liter, 10 mg of cefsulodin per liter, 62,000 IU of polymyxin B per liter, 40 mg of trimethoprim per liter, and 20 mg of sulfamethoxazole per liter to which either cycloheximide (20 or 40 mg/liter), vancomycin (20 or 40 mg/liter), or novobiocin (40 mg/liter) was added. After inoculation, the plates were incubated for 7 days as described above, and plates containing various selective agents were compared by evaluating the densities of the contaminating colonies on the plates. This procedure was repeated in a second trial by plating samples from 11 cattle onto media containing a basic combination consisting of 10 mg of cefsulodin per liter, 62,000 IU of polymyxin B per liter, 40 mg of trimethoprim per liter, and 20 mg of sulfamethoxazole per liter, to which either cycloheximide (10 mg/liter), cycloheximide (10 mg/liter) and amphotericin B (5 mg/liter), cycloheximide (10 mg/liter) and vancomycin (10 mg/liter), or cycloheximide (10 mg/liter) and nalidixic acid (10 mg/liter) were added.

Statistical analysis. For all experiments, plate counts were converted to $\log_{10}$ CFU per milliliter prior to statistical analysis by using the General Linear Models procedure of SAS (14). Duncan's multiple range test was used for mean separation ($P < 0.05$) in order to determine if there was a day effect for trials performed over several days and for samples collected from broth preparations after 24 and 48 h. If there were no day effects, data were combined, and log reductions ($\log_{10}$ CFU per milliliter on nonselective medium − $\log_{10}$ CFU per milliliter on medium containing selective agents) were compared.

RESULTS AND DISCUSSION

Growth on acidified agar. Because *H. pylori* survives in the acidic environment of the human stomach, our initial efforts were directed toward using low pH as a selective method to inhibit competing organisms. Statistical analysis revealed that there was no day effect in the experiment, so data obtained after incubation for 24 and 48 h were combined. *H. pylori* grew well at pH values of ≥5.5, but the colony size decreased significantly from 1.0 mm at pH 7.0 to 0.8 mm at pH 5.0. Additional experiments in which peptone water inocula rather than broth preparations were used showed that growth began to diminish at pH 5.5. The colony size decreased from 0.9 mm at pH 7.0 to 0.3 mm at pH 5.0. No growth was observed at pH values of ≤4.5.

Growth on acidified agar supplemented with urea. When urea was added at various concentrations, *H. pylori* grew well at pH 4.5 but not at pH 3.5 (Table 1). Compared to the experiment described above in which acidified agar was used, addition of urea resulted in good growth at pH 4.5, and at urea concentrations of 1, 5, and 10 mM the colonies were relatively large (diameters, 0.7 to 0.9 mm). The fact that adding urea allowed the bacterium to grow at lower pH values is consistent with other research which showed that survival of *H. pylori* improved at low pH values when urea was present (1, 8).

Growth in the presence of TTC. The mean amount of *H. pylori* recovered on HPSPA containing TTC or on HPSPA not containing TTC was 5.8 $\log_{10}$ CFU/ml, suggesting that TTC did not affect recovery of *H. pylori*. However, the mean colony diameter was significantly reduced ($P < 0.0001$) from 0.9 mm on HPSPA alone to 0.7 mm on HPSPA supplemented with

TABLE 1. Mean reductions in plate counts and colony sizes when *H. pylori* was plated from broth preparations onto media with various pH values and urea concentrations

pH	Urea concn (mM)	Reduction in plate counts ($\log_{10}$ CFU/ml)[a]	Colony diam (mm)
5.5	0	0.0 A[b]	0.9 A
5.5	0.1	0.1 A	0.7 AB
4.5	0	0.8 A	0.1 C
4.5	0.1	0.1 A	0.2 BC
4.5	1	+0.2 A[c]	0.7 A
4.5	5	0.0 A	0.9 A
4.5	10	+0.3 A	0.7 AB
3.5	10[d]	>3.1 B[e]	NG D[f]

[a] Reduction = mean $\log_{10}$ CFU per milliliter on HPSPA at pH 5.5 − mean $\log_{10}$ CFU per milliliter on HPSPA at a different pH and urea concentration.
[b] Values in the same column followed by the same letter are not significantly different ($P < 0.05$).
[c] A plus sign indicates that the plate counts were higher than the HPSPA plate counts at pH 5.5.
[d] Lower levels of urea (0, 0.1, 1, and 5 mM) were also tested, but no growth was observed.
[e] Below the limit of detection.
[f] NG, no growth.

TTC, indicating that growth was slightly inhibited. Although TTC has been used as a selective agent for *H. pylori* in one medium (15), we decided that TTC would not be used as a selective agent in this experiment because of the decrease in colony size.

Efficiency and selectivity of low pH plus various urea concentrations versus antibiotics as selective agents. Adding antibiotics (10 mg of vancomycin per liter, 10 mg of amphotericin B per liter, 10 mg of cefsulodin per liter, and 10 mg of trimethoprim per liter) did not affect colony size (diameter, 1.3 mm) and only slightly reduced the rate of recovery (0.4-log reduction) compared to standard HPSPA that did not contain selective agents. In contrast, decreasing the pH of the plating medium to 4.5 dramatically decreased the mean colony diameter from 1.3 mm at pH 7 to 0.6 mm at pH 4.5 in the presence of 10 mM urea. Various levels of urea (10, 20, or 40 mM) were compared to determine if one particular urea concentration enhanced growth of the bacterium at a low pH. Previous trials had shown that both 5 and 10 mM urea supported satisfactory growth of *H. pylori* at pH 4.5, but it was not known whether higher concentrations of urea would further enhance growth of the bacterium at a low pH. This experiment revealed that at pH 4.5, 10 mM urea was more beneficial than either 20 or 40 mM urea. Increasing the urea concentration to 20 or 40 mM decreased both the level of recovery and the growth rate of *H. pylori*. It is plausible that the very potent urease activity in the presence of high urea concentrations increased the pH to a value at which *H. pylori* does not grow well, a possibility which is consistent with other data describing the death of *H. pylori* at high urea concentrations (8). These data suggest that using antibiotics for selection may be more efficient for isolating *H. pylori* because the antibiotics tested inhibited bacterial growth less than low pH inhibited bacterial growth.

Visual evaluation of selective media inoculated with cattle samples revealed a large number of contaminants on all of the plates at pH 4.5, and many of the plates were covered with gram-negative spreaders. HPSPA supplemented with antibiotics allowed isolation of large numbers of *H. pylori* from all positive control samples and inhibited contaminating colonies much more effectively than low-pH (pH 4.5) HPSPA.

The only medium on which isolation of *H. pylori* from inoculated ground beef was efficient was HPSPA supplemented with antibiotics; on this medium 4.5 and 4.4 $\log_{10}$ CFU of *H. pylori* ATCC 43504 and ATCC 43629 per g, respectively, were recovered. The initial inoculation level was 5.4 $\log_{10}$ CFU/g, so an approximate 1-log reduction occurred during the inoculation and recovery process. On low-pH (pH 4.5) HPSPA containing various concentrations of urea (10, 20, or 40 mM) there were numerous contaminants growing on the plates, which prevented efficient isolation of *H. pylori*.

Since HPSPA supplemented with antibiotics (10 mg of vancomycin per liter, 10 mg of amphotericin B per liter, 10 mg of cefsulodin per liter, and 10 mg of trimethoprim per liter) recovered *H. pylori* from pure broth cultures more efficiently and minimized the growth of contaminating microorganisms more effectively, we decided that adding a combination of antibiotics is a more efficient selective method for isolating *H. pylori* from contaminated samples. Low pH (pH 4.5) and adding urea as the sole selective agents proved to be ineffective for isolating *H. pylori* from either bovine gastrointestinal samples or ground beef samples. While the antibiotic concentrations used in this experiment reduced the number of contaminants much more efficiently than low pH reduced the number of contaminants, these antibiotic concentrations still permitted a large number of contaminants to grow when samples from the bovine rumen were plated. Therefore, we compared additional antibiotic combinations that might enhance the selectivity of the medium.

Growth on HPSPA supplemented with additional combinations of antibiotics. Determining the combinations of antibiotics and the highest concentrations of these antibiotics that allowed good growth of *H. pylori* proved to be quite difficult because of interactions and synergy among multiple antibiotics and because of the fastidious nature of this bacterium. Numerous combinations of antibiotics and concentrations were compared in separate trials to determine the highest concentration of each antibiotic or antibiotic combination that still allowed acceptable growth of *H. pylori* (Table 2).

Trial 1 revealed that there were not significant differences in the rate of recovery or colony size among the antibiotic combinations used in this trial except for the combinations containing rifampin. When rifampin was added at a concentration of 2 mg/liter, the colony size was significantly reduced, and when it was added at a concentration of 4 mg/liter, *H. pylori* did not grow. Trial 2 revealed that there were not significant differences in the rate of recovery or colony size among the antibiotic combinations used except when cefsulodin was added at a concentration of ≥20 mg/liter. Adding 20 and 40 mg of cefsulodin per liter significantly reduced the rate of recovery by 1.6 and 1.8 log, respectively, and adding 40 mg of cefsulodin per liter significantly decreased the colony size.

Trial 3 revealed that there were not significant differences in the rate of recovery between HPSPA and any of the antibiotic combinations used, but colony size decreased with all of the antibiotic combinations. The most dramatic decreases in colony size occurred after the addition of either 124,000 IU of polymyxin B per liter or 120 mg of trimethoprim per liter and 80 mg of sulfamethoxazole per liter, which reduced the colony diameter from 1.2 mm to 0.3 and 0.4 mm, respectively. All other antibiotic combinations decreased the colony diameter from 1.2 mm to 0.5 to 0.6 mm, indicating that the bacterium was moderately inhibited. Initially, the reductions in colony size were considered acceptable when they were coupled with the fact that the antibiotic combinations did not reduce the rate of recovery. However, subsequent experiments revealed that a concentration of novobiocin as low as 2.5 mg/liter dramatically reduced the growth of *H. pylori*.

Trial 4 showed that no growth of *H. pylori* occurred when we used antibiotic combinations containing 10 mg of novobiocin

TABLE 2. Mean reductions in plate counts and colony sizes when *H. pylori* was plated from broth preparations onto media containing various combinations of antibiotics[a]

Trial	Antibiotic(s)	Reduction in plate counts (log_{10} CFU/ml)[b]	Colony diam (mm)
1[c]	None	0.0 A[d]	1.3 A
	VACT, nalidixic acid (10 mg/liter)[e]	0.0 A	1.2 A
	VACT, polymyxin B (62,000 IU/liter)	0.0 A	1.2 A
	VACT, cycloheximide (10 mg/liter)	0.0 A	1.3 A
	VACT, rifampin (2 mg/liter)	2.5 B	0.1 B
	VACT, rifampin (4 mg/liter)	NG[f]	NG
2	None	0.0 A	1.2 A
	VAPT, cefsulodin (10 mg/liter)[g]	0.2 A	1.1 AB
	VAPT, cefsulodin (20 mg/liter)	1.6 B	1.0 B
	VAPT, cefsulodin (40 mg/liter)	1.8 B	0.8 C
	VAPT, cefsulodin (10 mg/liter), sulfamethoxazole (8 mg/liter)	0.1 A	1.1 AB
	VAPT, cefsulodin (10 mg/liter), sulfamethoxazole (16 mg/liter)	0.0 A	1.1 AB
3	None	0.0 A	1.2 A
	VACPTS, novobiocin (10 mg/liter)[h]	0.4 A	0.7 B
	VACPTS, novobiocin (20 mg/liter)	0.2 A	0.6 B
	VACTS, polymyxin B (124,000 IU/liter)[h]	0.2 A	0.3 C
	VACPS, trimethoprim (60 mg/liter)[h]	0.7 A	0.6 B
	VACP, trimethoprim (120 mg/liter), sulfamethoxazole (80 mg/liter)[h]	0.4 A	0.4 C
4	None	0.0 A	0.9 A
	Polymyxin B (62,000 IU/liter)	0.4 A	1.2 B
	Cefsulodin (10 mg/liter), polymyxin B (62,000 IU/liter)	0.9 A	1.1 B
	CPTS[i]	1.0 A	1.0 A
	CPTS, novobiocin (10 mg/liter)	NG	NG
	CPTS, novobiocin (10 mg/liter), amphotericin B (10 mg/liter)	NG	NG
5	None	0.0 A	0.9 A
	VCPTSC[j]	0.1 A	0.8 B
	VCPTSC, nalidixic acid (10 mg/liter)	0.1 A	0.7 B
	VCPTSC, nalidixic acid (10 mg/liter), amphotericin B (2.3 mg/liter)	0.1 A	0.7 B
	VCPTSC, nalidixic acid (10 mg/liter), amphotericin B (4.5 mg/liter)	0.2 A	0.7 B
	Novobiocin (2.5 mg/liter)	0.2 A	0.5 C
6	None	0.0 A	0.8 A
	CPTSC[k]	0.1 A	0.8 A
	CPTSC, amphotericin B (2.5 mg/liter)	0.1 A	0.8 A
	CPTSC, amphotericin B (5 mg/liter), vancomycin (10 mg/liter)	0.1 A	0.8 A
	CPTSC, amphotericin B (5 mg/liter), vancomycin (10 mg/liter), nalidixic acid (10 mg/liter)	0.2 A	0.7 A
7	None	0.0 A	0.9 A
	VACPTS[l]	0.4 A	0.7 B

[a] HPSPA (pH 7.0) was the standard plating medium, and various combinations of antibiotics were added to this medium.
[b] Reduction = mean log_{10} CFU per milliliter on HPSPA without antibiotics − mean log_{10} CFU per milliliter on HPSPA containing antibiotic(s).
[c] Statistical analysis revealed that there was a day effect, so data from each trial were evaluated separately.
[d] Values in the same column for the same trial followed by the same letter are not significantly different ($P < 0.05$).
[e] In trial 1, VACT consisted of 10 mg of vancomycin per liter, 10 mg of amphotericin B per liter, 10 mg of cefsulodin per liter, and 10 mg of trimethoprim per liter.
[f] NG, no growth.
[g] In trial 2, VAPT consisted of 10 mg of vancomycin per liter, 10 mg of amphotericin B per liter, 62,000 IU of polymyxin B per liter, and 20 mg of trimethoprim per liter.
[h] In trial 3, VACPTS consisted of 10 mg of vancomycin per liter, 10 mg of amphotericin B per liter, 10 mg of cefsulodin per liter, 62,000 IU of polymyxin B per liter, 20 mg of trimethoprim per liter, and 20 mg of sulfamethoxazole per liter; VACTS consisted of 10 mg of vancomycin per liter, 10 mg of amphotericin B per liter, 10 mg of cefsulodin per liter, 20 mg of trimethoprim per liter, and 20 mg of sulfamethoxazole per liter; VACPS consisted of 10 mg of vancomycin per liter, 10 mg of amphotericin B per liter, 10 mg of cefsulodin per liter, 62,000 IU of polymyxin B per liter, and 20 mg of sulfamethoxazole per liter; and VACP consisted of 10 mg of vancomycin per liter, 10 mg of amphotericin B per liter, 10 mg of cefsulodin per liter, and 62,000 IU of polymyxin B per liter.
[i] In trial 4, CPTS consisted of 10 mg of cefsulodin per liter, 62,000 IU of polymyxin B per liter, 20 mg of trimethoprim per liter, and 10 mg of sulfamethoxazole per liter.
[j] In trial 5, VCPTSC consisted of 10 mg of vancomycin per liter, 10 mg of cefsulodin per liter, 62,000 IU of polymyxin B per liter, 40 mg of trimethoprim per liter, 20 mg of sulfamethoxazole per liter, and 10 mg of cycloheximide per liter.
[k] In trial 6, CPTSC consisted of 10 mg of cefsulodin per liter, 62,000 IU of polymyxin B per liter, 20 mg of trimethoprim per liter, 10 mg of sulfamethoxazole per liter, and 10 mg of cycloheximide per liter.
[l] In trial 7, VACPTS consisted of 10 mg of vancomycin per liter, 5 mg of amphotericin B per liter, 10 mg of cefsulodin per liter, 62,000 IU of polymyxin B per liter, 40 mg of trimethoprim per liter, and 20 mg of sulfamethoxazole per liter.

per liter. There were not significant differences in the rates of recovery obtained with the other antibiotic combinations used in this trial, and colony size was not significantly decreased by 10 mg of cefsulodin per liter, 62,000 IU of polymyxin B per liter, 40 mg of trimethoprim per liter, and 20 mg of sulfamethoxazole per liter. These data indicate that adding novobiocin to the other four antibiotics inhibited growth of *H. pylori*.

In trial 5 there were not significant differences in the rate of recovery between HPSPA without antibiotics and HPSPA containing any of the antibiotic combinations. The colony diame-

ter was reduced by 0.1 to 0.2 mm by most of the antibiotic combinations; the only exception was 2.5 mg of novobiocin per liter, which decreased the colony diameter from 0.9 to 0.5 mm. Although the reductions in colony size were statistically significant, all of the antibiotic combinations except 2.5 mg of novobiocin per liter still allowed relatively large colonies (diameter, 0.7 to 0.8 mm) to grow. Coupled with the fact that the antibiotic combinations did not reduce the rate of recovery, the minor reductions in colony size were considered acceptable. These data provide further evidence that novobiocin, even at a low level (2.5 mg/liter), inhibits the growth of *H. pylori*; this conclusion is consistent with the results of trial 4 and 3 additional trials (data not shown), in which novobiocin at a concentration of ≥10 mg/liter prevented the growth of *H. pylori*. We were reluctant to eliminate novobiocin as a selective agent because it dramatically decreased the numbers of contaminants in bovine samples and because the trial 3 results indicated that *H. pylori* was somewhat resistant to novobiocin. However, on the basis of the data from four trials, we concluded that *H. pylori* is sensitive to novobiocin even at low concentrations, so novobiocin was eliminated from consideration as a selective agent.

Trials 6 and 7 provided final confirmatory evidence that *H. pylori* grows well in the presence of 10 mg of vancomycin per liter, 5 mg of amphotericin B per liter, 10 mg of cefsulodin per liter, 62,000 IU of polymyxin B per liter, 40 mg of trimethoprim per liter, and 20 mg of sulfamethoxazole per liter.

Selectivity of HPSPA containing additional antibiotics when it is inoculated with rumen and abomasum samples from cattle. All of the antibiotic combinations used in trial 1 limited the growth of contaminants, and individual colonies were easily identified. Media containing vancomycin (20 or 40 mg/liter) instead of cycloheximide contained fewer contaminants and allowed easier identification of individual colonies. These combinations of antibiotics also allowed successful isolation of numerous colonies of *H. pylori* from most positive control samples. This trial revealed that antibiotic combinations containing 20 mg of vancomycin per liter, 10 mg of amphotericin B per liter, 10 mg of cefsulodin per liter, 62,000 IU of polymyxin B per liter, 20 mg of trimethoprim per liter, and 20 mg of sulfamethoxazole per liter efficiently prevent growth of contaminants but still allow outgrowth and isolation of *H. pylori*.

In trial 2 HPSPA containing 10 mg of cefsulodin per liter, 62,000 IU of polymyxin B per liter, 40 mg of trimethoprim per liter, and 20 mg of sulfamethoxazole per liter permitted growth of several contaminants, but individual colonies of *H. pylori* were still easily identified and isolated. Adding 10 mg of nalidixic acid per liter or 10 mg of cycloheximide per liter to the basic antibiotic combination did not improve the selectivity. However, adding 5 mg of amphotericin B per liter to the basic antibiotic combination enhanced the selectivity, and adding 10 mg of vancomycin per liter further enhanced the selectivity. Thus, the combination consisting of 10 mg of vancomycin per liter, 5 mg of amphotericin B per liter, 10 mg of cefsulodin per liter, 62,000 IU of polymyxin B per liter, 40 mg of trimethoprim per liter, and 20 mg of sulfamethoxazole per liter eliminated growth of a large number of contaminants but still allowed successful isolation of numerous colonies of *H. pylori* from positive control samples, which confirmed the results obtained in trial 1.

The medium described in this paper was designed and tested for the ability to recover *H. pylori* from cattle samples. Because of the fastidious nature of this organism, defining selective agents that inhibit large numbers of contaminants while still allowing *H. pylori* to grow well is extremely challenging, yet the final selective HPSPA formulation, which includes 10 mg of vancomycin per liter, 5 mg of amphotericin B per liter, 10 mg of cefsulodin per liter, 62,000 IU of polymyxin B per liter, 40 mg of trimethoprim per liter, and 20 mg of sulfamethoxazole per liter, has proven to be highly selective while still permitting growth of large colonies of *H. pylori*. This medium, which is effective for isolating *H. pylori* from inoculated cattle and beef samples, may also be useful for recovering this pathogen from both human and environmental samples that may be contaminated with large numbers of competing microorganisms.

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