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DEVELOPMENT AND APPLICATION OF A PLATING MEDIA FOR DETECTION OF *HELICOBACTER PYLORI* IN WATER

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ABSTRACT

In the U.S. alone, about 5,000,000 people are diagnosed annually with ulcers, 1,000,000 are hospitalized, 40,000 undergo surgery, and 6,500 die from ulcer-related complications (Poms, 2001: Levin, 1998). Once thought to be a result of stress and/or diet, ulcers are now almost exclusively attributed to infection with the bacteria *Helicobacter pylori*. Laboratory diagnosis of *H. pylori* has become a standard procedure in the management of dyspeptic patients. Although transmission of the organism through the fecal/oral route is the assumed infection route, the possible mechanisms of human infection such as food, person to person contact, water or fomites are not clearly understood. There are a few reports in the literature suggesting transmission of *H. pylori* to humans via groundwater (Hegarty, 1999; Hulten, 1995 & 1998). Methods of detection used in those studies were relatively complex and costly (polymerase chain reaction; immunomagnetic separation) and unfortunately, didn't determine if the detected organisms were in fact viable or infectious. The work reported here focused on the development of a microbiological plating media that selects viable *H. pylori* organisms from samples containing mixed microbial populations, which could then be used for routine screening of ground and/or surface water for the presence of *H. pylori*. Efforts have resulted in a media formulation that allows the growth of *Helicobacter* while subsequently excluding common waterborne microbial background contaminants such as gram positive cocci and bacilli, enterobacteriaceae, gram negative bacilli, fungi, and pseudomonads. The laboratory-tested plating media was used to survey a cross section of Wisconsin groundwaters to further evaluate the efficacy of the media for recovering *H. pylori* from water samples and to begin a data base of *H. pylori* occurrence.

INTRODUCTION

A scientific breakthrough occurred in 1982 when J. R. Warren and B. Marshall isolated a bacterium and showed that it caused gastritis and stomach ulcers that affect millions of humans worldwide (Marshall, 1984). Today that claim has been proven to the extent that the National Institute of Health recommends treatment with antibiotics for all patients with peptic ulcers, which are almost exclusively attributed to infection with the bacteria *Helicobacter pylori* (Graham, 1991). The scope of gastric illnesses around the world is vast. In the U.S. alone, estimates as high as 50% of adult Americans carry the pathogen, most asymptomatically, and in less-developed countries human carriers represent up to 90% of the populations (Munangi, 1997).

The source of human infection is not yet known and until recently, the natural reservoir for *H. pylori* was thought to be the human gastrointestinal tract (Axon, 1996). However, isolation of *Helicobacter* from non-human sources such as livestock (Vaira, 1992), domestic cats

(Otto, 1994), and vegetables (Hopkins, 1993) prompted researchers to look at environmental sources as vectors for human infection. Previous efforts suggest the presence of *H. pylori* in ground water, surface water, and other drinking water (Mazari-Hiriart, 2001; Yingzhi, 2002; Hegarty, 1999; Hulten, 1998; Hulten, 1995; Shahamat, 1993), therefore implying a waterborne route of transmission to humans. The complex methods used in these studies (polymerase chain reaction; immunomagnetic separation; autoradiography; enzyme immunoassay) are expensive to run, subject to numerous interferences and do not differentiate between viable and non-viable organisms. A low cost and effective test to isolate viable *H. pylori*, similar to selective media used for *Salmonella* or *E. coli*, from ground and surface water would enable the drinking water industry to routinely screen samples. This study focused on efforts towards development of a plating media that selects viable *H. pylori* from real world water samples containing mixed microbial populations.

MATERIALS & METHODS

Media development

Bacterial strain management

Five clinical infection (no environmental isolates are available in U.S. or European type culture collections) *Helicobacter pylori* strains were obtained from the American Type Culture Collection (ATCC; Manassas, VA) or the Wisconsin State Laboratory of Hygiene culture collection (WSLH; Madison, WI; Table 1) and cultured on Brain Heart Infusion agar (BHI; Becton Dickinson, Sparks, MD) supplemented with 7% calf serum. The plates were incubated under a microaerophillic gas mixture (5% $C0_2$; 10% H_2; 85% N_2; Praxair, Inc., Danbury, CT) at 37°C. Frozen stock cultures were prepared by picking isolated colonies from agar plates and homogenizing in sodium phosphate buffer to about 10^7 colony forming units (cfu)/ml using a McFarland nephelometer. Each of the five isolates was then frozen in BHI broth containing 10% glycerol. Sufficient quantities were prepared to complete the entire study in order to avoid multiple passing of strains, which can sometimes lead to phenotypic variability. The non-*Helicobacter* bacteria used to create the complex spiked water samples (Table 1) were obtained from the WSLH culture collection, grown on BHI agar slants at 35°C and then stored at 4°C for up to 3 weeks before re-culturing. The bacteria are listed in table 1.

TABLE 1. Bacterial strains and sources used in preparing water samples containing known levels of contaminants.

Helicobacter pylori	[a]ATCC 43504
Helicobacter pylori	ATCC 700392
Helicobacter pylori	ATCC 49503
Helicobacter pylori	[b]WSLH 95-10882
Helicobacter pylori	WSLH 409013
Acinetobacter	WSLH
Aeromonas	WSLH
E. coli	WSLH
Pseudomonas aeruginosa	WSLH
Enterobacter cloacea	WSLH
Enterococcus faecalis	WSLH
Bacillus	WSLH

[a] American Type Culture Collection.
[b] Wisconsin State Laboratory of Hygiene culture collection.

Preparation of conventional media

Conventional dehydrated media preparations were chosen for selection of *H. pylori* from mixed microbial populations based on the published clinical literature. The five types of media chosen for evaluation were BHI + 7% calf serum (Osaki, 1998), Brucella Agar (Poms, 2001), Campylobacter Agar Kit Skirrow (Corry, 1995), Columbia Blood Agar Base (Baronn, 1994; Becton Dickinson, Sparks, MD), and HPSPA (Jiang, 2000; Stevenson, 2000). Table 2. compares ingredients of these media to demonstrate the common and unique features of each. The inclusion of either *Helicobacter pylori* selective supplement (Oxoid Limited, Hampshire, England) or *Campylobacter* selective supplement S (Becton Dickinson) provided antibiotics to prevent background while permitting *H. pylori* growth. Positive control consisted of the five conventional media without the selective supplements while negative controls consisted of uninoculated plates. All media were prepared according to manufacturer's or authors' instructions.

TABLE 2. Formulations of conventional media used to culture and select *Helicobacter pylori.*

Brain Heart Infusion	Brucella Agar	Columbia Agar	[a]HPSPA	Campylobacter Agar
10 g proteose peptone	10 g peptamin	10 g pantone	15 g spec. peptone	15 g proteose peptone
NA beef heart inf.	10 g tryptone	10 g bitone	2 g porcine mucin	2.5. ml liver digest
NA calf brains inf.	2 g yeast extract	3 g beef heart dig.	5 g yeast extract	5 g yeast extract
2 g dextrose	1 g dextrose	1 g dextrose	5 g beef extract	
5 g NaCl	5 g NaCl	5 g NaCl	5g NaCl	5g NaCl
15 g agar	15 g agar	15 g agar	15 g agar	12 g agar
2.5 g disodium phos.	0.1 g Na bisulfate		0.5 g ferrous sulfate	
70 ml calf serum w/fe			70 ml calf serum w/fe	
			0.6 g urea	
			0.5 g Na pyruvate	
[b]selective supplement	selective supplement	selective supplement	selective supplement	[c]selective supplement

[a] Formula published by Jiang and Doyle. J. Clin. Microbiol. 2000.
[b] Vancomycin (10 mg/L), Cefsulodin (5 mg/L), Trimethoprim (5 mg/L), and Amphotericin B (5 mg/L).
[c] Vancomycin (10 mg/L), Trimethoprim (5 mg/L), and Polymyxin B (2500 IU/L)

Initial conventional test media screen

Evaluation of the efficacy of the conventional media formulations used in clinical microbiology laboratories was carried out as follows. Each strain listed in Table 1 was separately cultured on each of the five media listed in Table 2 in order to evaluate the growth and inhibition spectra of individual formulations. First, pure colonies of each strain were picked from solid growth media (BHI agar plates) and homogenized in sodium phosphate buffer (4%). Serial dilutions of each pure homogenate were immediately spread (0.1ml/plate) onto each type of solid media with and without selective supplement added. All plates were incubated under microaerophillic conditions at 37°C for up to seven days and examined to determine the presence or absence of colonies on the media compared to the positive control of a non-selective media.

Selectivity of media formulations for *H. pylori* from samples spiked with a cocktail of non *H. pylori* bacteria.

Well water samples containing native flora (Table 5; identified using API 20 E identification system; Biomerieux Vitek, Inc., Hazelwood, MO) were further spiked with the seven strains of background bacteria described above, and the *H. pylori*, to represent a highly contaminated water sample (10,000 cells per strain per 100 mls). The spiked water sample was then serially diluted and 0.1 ml of each dilution was spread onto duplicate plates of each of the

five conventional media, supplemented with either Helicobacter pylori or Campylobacter selective supplement (Table 2). Positive controls were the same as described above. All plates were incubated in microaerophillic atmosphere at 37°C for up to 7 days to provide the optimal environment for culturing *Helicobacter.*

Formulation of enhanced selectivity (HP) media

HP media preparation requires a sequential addition of components. The mixture of special peptone, beef extract, yeast extract, NaCl, phenol red, agar, and water was autoclaved for 20 minutes at 121°C and then tempered to 50°C. Then calf serum with iron, antibiotics (vancomycin trimethoprim, cefsulodin, Amphotericin B, and Polymixin B), and urea were aseptically added with constant stirring. Finally 0.8 ml of 1N HCl was added drop-wise to the media as color changed from red to yellow/orange (final pH 5.7 @ 45°C, pH 6.0 @ 22°C). The media was then poured into petri plates.

Table 3. Media components evaluated over a range of concentrations to develop the final HP formulation.

Component	**Range tested** per liter	[a] **optimum level**
Porcine mucin	0 – 4 g	O
Ferrous sulfate	0 – 500 mg	O
Na pyruvate	0 – 500 mg	O
Polymixin B	0 – 4000 units	3500 units
Amphotericin B	0 – 7.5 mg	7.5 mg
Vancomycin	0 – 10 mg	10 mg
Trimethoprim	0 - 5 mg	5 mg
Cefsulodin	0 - 5 mg	5 mg
Urea	0 – 1 mg	600 mg
Phenol red	0 – 200 mg	100 mg
1 N HCl	0 – 2 ml	0.8 ml

[a] optimum level for recovering *H. pylori* while inhibiting background flora.

Well sample testing

About three hundred fifty private drinking water wells across Wisconsin were screened using HP agar, immunomagnetic separation (IMS), and/or fluorescent antibody staining (FA) for the presence of *H. pylori.* The samples were grouped into two types, designated random or requested.

Random samples were collected by taking aliquots from samples sent to the WSLH Water Bacteriology Department for routine coliform testing. For this group, fifty to one hundred ml aliquots were centrifuged at 2500 x g for 15 minutes and then all but the bottom 5 mls of supernatant was carefully siphoned away. 0.5 ml aliquots of the resulting 5 mls (or dilutions thereof) were then spread plated onto HP agar plates and incubated in a microaerophilic environment at 37°C for up to 7 days. Colonies appearing after 3 days with reddish "halos" (urease +), 1-2 mm in diameter, catalase and urease positive were considered positive. Subsequent microscopic examination showing helical, horseshoe, and/or coccoid morphology was used for verification. About 275 wells were represented in the grouping of random samples.

The requested sample group consisted of wells where a specific request to test for *H. pylori* was received from the well owner. These requests most likely resulted from the fact that a resident in the home served by the well had suffered from an *H. pylori* infection. For this group, 0.5 liter was concentrated to 5 ml via centrifugation. Four mls of the final concentration was spread onto HP agar plates (0.5 ml per plate) and incubated as described. For the requested sample group, two additional methods were employed in an attempt to recover *H. pylori*. In addition to the culture another 1 ml aliquot was assayed using immunomagnetic separation (IMS). Briefly, the 1 ml aliquot is mixed with microscopic magnetic beads (Dynabeads M-450, Dynal ASA, Oslo, Norway) coated with antibodies specific to *H. pylori*. The quantity of beads and duration of contact mixing time was determined by manufacturer's recommendations to provide the optimal exposure of capture beads to target organism. During this process the bacteria immunologically attach to the magnetic particles. A magnetic tube rack is then used to retrieve the beads and attached *H. pylori*. The retrieved beads are then plated onto the HP agar. A third procedure involving direct fluorescent antibody staining of a filtered sample was also employed as a non culture method control on some samples. For the fluorescent antibody (FA) staining, 0.5 liter of sample water was passed through a 0.4 micron pore size filter to capture *H. pylori*. The filter was treated with fluorescent stain (IgG Fluor, Chemicon International, Temecula, CA) attached to *H. pylori*-specific antibodies (Biodesign International, Saco, ME) that attach to the target cell if present. Cells reflecting green under fluorescent light microscope and helical, horse-shoe, or folded shape were considered *H. pylori* (note: non-viable as well as viable cells will fluoresce). IMS was used when samples contained visibly high particulate matter and FA staining was used to assay clear water.

RESULTS & DISCUSSION

Media evaluation

Growth of pure cultures on conventional media.

Table 2 lists the five conventional media formulations evaluated. Each of these media was evaluated for its ability to recover *H. pylori* from among a population of 7 spiked strains of bacteria and various indigenous strains contained in a sample of well water (Table 5). As expected, media without the antibiotic supplement allowed the growth of all organisms tested. The rapid growth of the non *H. pylori* bacteria covered the plates precluding any chance of detecting the slow growing *H. pylori* organisms. The addition of selective supplements provided some measure of selective pressure, however, some of the organisms (*Acinetobacter, E. coli, Flavobactrum, Pasteurella, Ochrobactrum*) were not inhibited and unacceptable levels of overgrowth still occurred. The selectivity profiles were identical among the five conventional media formulations, although it was noted that *H. pylori* colonies formed most rapidly (84 hours) on HPSPA media.

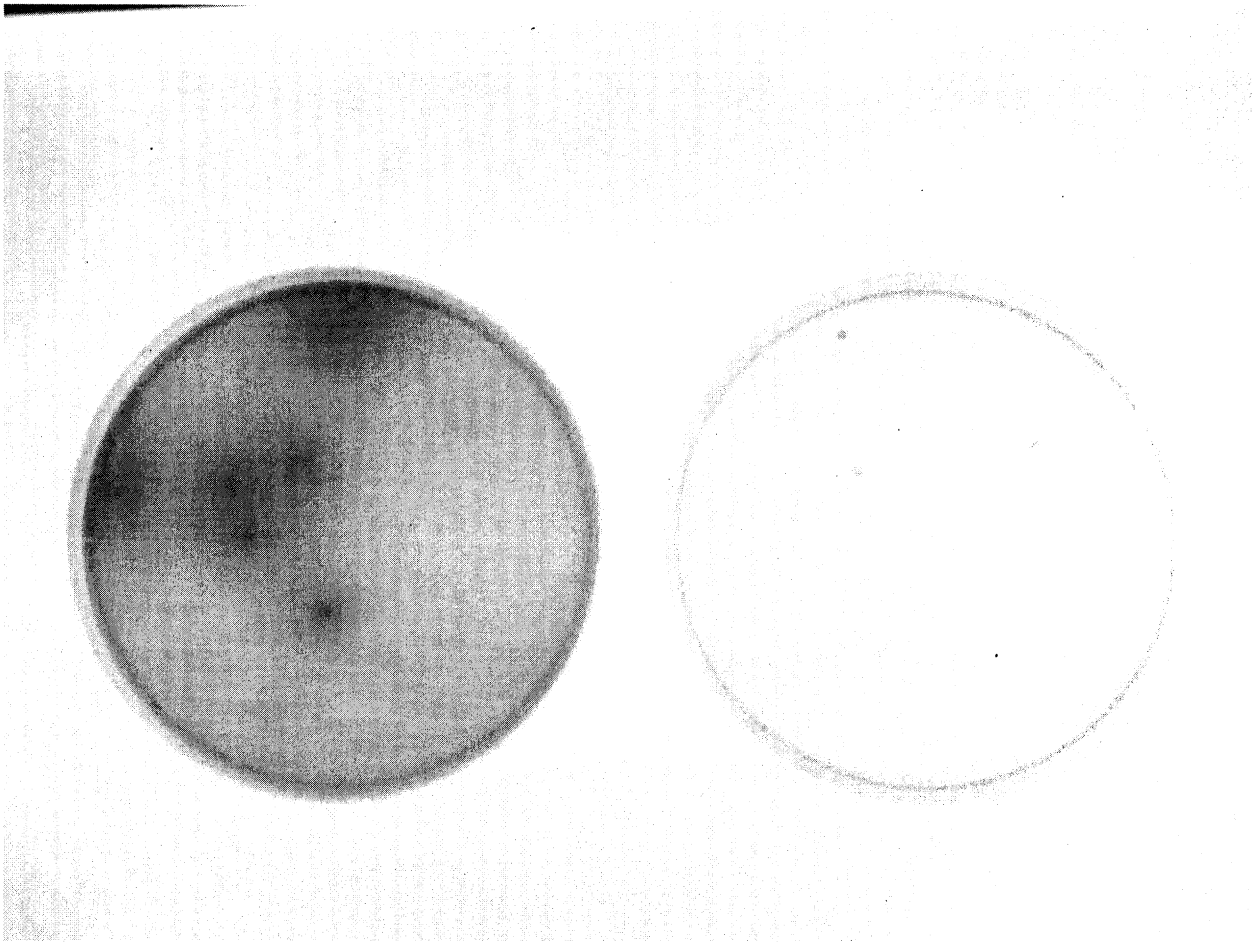
Since the selectivity of the five conventional media proved inadequate to isolate *H. pylori* from the complex flora water samples it was determined that an enhanced selective media would be required. To develop the formula for this new media, components were individually evaluated to determine their contribution to the selective and nutritive properties necessary to isolate *H. pylori* from a mixed population of microbial contaminants. Some nutritive components (yeast extract, beef extract, special peptone [Oxoid], NaCl) were incorporated at conventional concentrations without further evaluation. In order to develop a media with enhanced selectivity for *Helicobacter*, a list of selective, nutritional, and differential components were evaluated (Table 3). The properties of an improved formulation would include a broader inhibition

spectrum that includes *E. coli, Acinetobacter, Flavobactrum, Pasteurella,* and *Ochrobactrum*, as well as inhibition of molds.

The growth of *H. pylori* was not enhanced by the presence of the various concentrations of ferrous sulfate, sodium pyruvate, or porcine mucin applied. Therefore these components were omitted from the final formulation. However, more rapid colony formation on HPSPA media was attributed to the presence of Special Peptone and calf serum with iron, so these remained as nutritional components. Increasing the levels of vancomycin or Cefsulodin above 10 mg/L resulted in retarding the growth of *Helicobacter*, as did increasing trimethoprim above 5 mg/L. Conversely, reducing below these levels allowed background contamination. Therefore these concentrations (as contained in the selective supplement) were considered optimal. Increasing the level of amphotericin B from 5 to 7.5 mg/L was adequate to broaden the spectrum to inhibit all apparent background bacteria. The addition of polymyxin B at 3500 International Units (IU) appreciably reduced the occurrence of mold contaminants while having little deleterious effects on *H. pylori* colony development.

The novel color indicator system to differentiate *H. pylori* among non-urease producers greatly enhanced the utility of the media (Figure 1). Colony growth and subsequent urease production reduced urea to ammonium and bicarbonate, thus neutralizing a discreet area around each colony. This area of neutralization was marked by a zone of red around the colony as pH of the media changed from about 6.0 to >7.5. Incorporating the color indicator accelerated presumptive identification of *H. pylori* colonies by at least 12 hours (from 84 down to 72 hours). The final formulation of HP agar is listed in Table 4.

FIGURE 1. Helicobacter pylori on HP or Brain Heart Infusion agar plates.



HP agar Brain Heart Infusion agar

Table 4. Formulation for one liter of HP media.

Component	Amount
Special peptone	12 g
Yeast extract	5 g
Beef extract	5 g
NaCl	5 g
A calf serum with Fe	70 ml
Polymixin B	3500 units
Amphotericin B	7.5 mg
Vancomycin	10 mg
Trimethoprim	5 mg
Cefsulodin	5 mg
Urea	600 mg
Phenol red	100 mg
1 N HCl	0.8 ml
Agar	15 g
Distilled water	1 liter

[a] aseptically added post-autoclaving and tempering to 50°C.
[b] 1 N HCl added drop-wise to cause color change from red to yellowish.

Selectivity of modified HP media

The local well water used in these experiments contained about 400 native heterotrophic bacteria per 100 ml, some of which were identified *as Flavobactrum, Serratia, Citrobacter, Pasteurella, Ochrobactrum,* and *Rahnella*, using the API 20 E identification system (Table 5). In addition to the native flora, seven additional strains of bacteria and an *H. pylori* cocktail were added at levels of about 10,000 cfu/ 100 mls each. Dilutions of the adulterated well water were then plated onto BHI agar w/ 7% calf serum (positive control) and HP agar. The plates were incubated under microaerophillic conditions for up to 7 days and monitored for colony development. The positive control media without the selective agents became overgrown with bacterial colonies within 24 hours of incubation. The HP agar plates however contained only colonies of *H. pylori* over the seven day incubation. Colonies were presumptively recognizable within 72 $\pm$ 8 hours (c.a. 14% less) because of the pH indicator and resultant red halo indicating urease production. In addition to shorter incubation periods, interference from background bacteria and/or molds was not problematic because of the increased levels of antibiotics.

TABLE 5. Growth (+) and inhibition (none) of water related bacterial strains on conventional media with and without *Helicobacter pylori* selective supplements, and on newly formulated HP agar media.

Bacterial strains	Solid media		
Supplementary	without [a]supplement	with supplement	[b]HP formula
Acinetobacter	+	+	none
Aeromonas	+	none	none
Bacillus	+	none	none
E. coli	+	+	none
Enterobacter cloacea	+	none	none
Enterococcus faecalis	+	none	none
Helicobacter	+	+	+
Pseudomonas aeruginosa	+	none	none
Flora indigenous to well water			
Flavobactrum	+	+	none
Serratia	+	none	none
Citrobacter	+	none	none
Pasteurella	+	+	none
Ochrobactrum	+	+	none
Rahnella	+	none	none

DISCUSSION

Five conventional media formulations showed that all were comparably nutritious in culturing *H. pylori* as well as the native and added background organisms (Table 5). However, developing an acceptable selective media for *H. pylori* in water presented the classical microbiological problem of finding a media that is nutritionally rich enough to resuscitate and grow this fastidious organism while managing to inhibit the growth of all the other organisms found in water samples. *Helicobacter* is a relatively slow-growing bacteria usually requiring about 4 days to develop discernible colonies. This genus can easily be overgrown on solid media by robust strains that grow readily within 24 hours, and thereby conceal the presence of the pathogen. The antibiotic resistance spectrum of *H. pylori* is well defined but the concentrations in media vary widely depending on the matrix in which the research is done. For example, trimethoprim and polymyxin B were used at 5 mg and 3,500 IU, respectively, to isolate *H. pylori* in a water matrix (Penner, 2000) but were increased to 40 mg and 62,000 IU for selectivity in cattle stomach (Stevenson, 2000). In this study, we focused on antibiotic levels established by commercial vendors (Oxoid, Becton Dickinson) and adjusted these components in order to achieve acceptable results.

Commercial supplements allowed some background strains (*E. coli, Acinetobacter, Flavobactrum, Pasteurella,* and *Ochrobactrum*) to grow during the moist, warm (37°C), and relatively lengthy (up to 7 days) incubation. In spite of extraordinary care in maintaining sterility, molds also frequently developed and overwhelmed the plates. Thus some of the selective components were increased after individually evaluating each antibiotic. Increasing amphotericin from 5 to 7.5 mg/L and adding polymyxin B at 3,500 IU/L was adequate to solve the contamination problems, while having no apparent deleterious effects on *H. pylori* growth.

Although Jiang and Doyle (2000) reported that porcine mucin, ferrous sulfate, and sodium pyruvate were important in improving recovery of *H. pylori* cells, there was no appreciable difference in the presence or absence of these components in this study. The concept of using the characteristic urease production appears in a clinical assay (CLO rapid urease test; Delta West Pty Ltd, Bentley, Australia) but has not been previously reported in solid media designed for culturing *Helicobacter.* The addition of this color system substantially reduces the time required (about 14%) to grow colonies to the point of presumptive visual identification. Even on HP media plated with heavy bacterial loads, the occasional development of a background colony was easily differentiated from *H. pylori.*

In summary, the HP formulation provides a media with superior selectivity for *H. pylori* from mixed microbial populations in water and reduces the time required to complete the assay.

Well water survey

Random samples

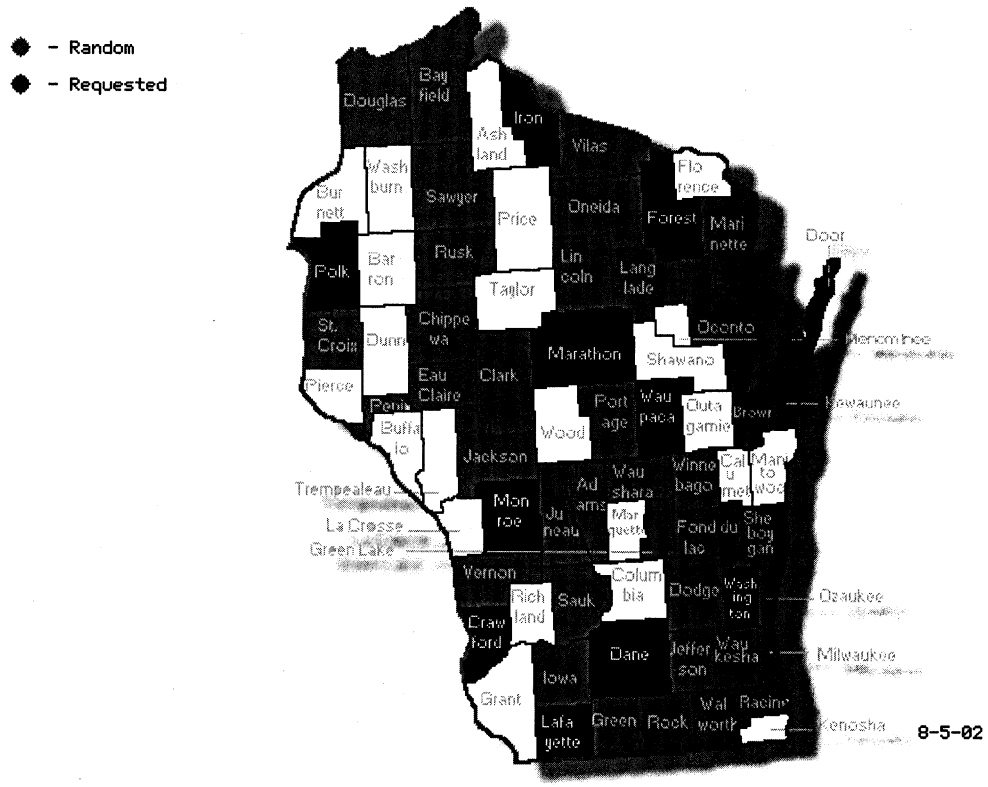
About 275 samples representing private Wisconsin wells were randomly chosen from those sent to the Wisconsin State Laboratory of Hygiene for routine coliform testing. The samples were centrifuged and reduced volumes were plated onto HP media. Assays were accompanied with positive (reverse osmosis water spiked with *H. pylori*) and negative (non-spiked water) controls to monitor performance. *H. pylori* was not detected in any of these samples which were incubated for 7 days at 37°C. About 95% of the plates were barren following incubation, which demonstrates that HP agar is effective in inhibiting growth of background contamination, even though some of these samples contained heavy flora.

Requested samples

About 75 wells were screened for the presence of *H. pylori* at the request of owners suffering from peptic ulcers or related symptoms. HP agar in addition to immunomagnetic separation and/or fluorescent antibody staining were used to screen these samples. In addition to *Helicobacter* screening, information regarding the history of the wells, well location, consumers' symptoms, and coliform/*E. coli* presence in the sample was recorded.

Although this subset of samples was relatively unsanitary (up to 10^4 heterotropic bacteria/ml), with about 25% positive for coliforms and one containing *E. coli*, none were positive for *Helicobacter.* Demographics of these wells reveal that 95% were neither tested nor disinfected since being drilled. The locations of the wells were widely distributed across the state (Figure 2) and the quality of water ranged from clear and sterile to turbid and unsafe. The results from HP agar were confirmed by two supporting methodologies which are accepted as reliable and sensitive by previous research groups. Therefore, interpretation of these data must conclude that water, as far as Wisconsin, U.S.A. well water is concerned, is not a likely vector for the transmission of *H. pylori* to humans.

FIGURE 2. Wisconsin counties containing private wells sampled randomly (blue) or requested by residents suffering gastrointestinal problems (black).



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