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Isolation and Genotyping of Helicobacter pylori from Untreated Municipal Wastewater

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For this study, we isolated Helicobacter pylori from wastewater by a series of steps beginning with immunomagnetic separation and cell culture. After Gram staining and three standard microbial tests, the 16S rRNA sequences of a total of 23 out of 37 putative H. pylori isolates were verified by PCR. Eleven H. pylori isolates were genotyped and fell into four vacA classes: those with the vacA allelic variants s1a and m1, s1b and m1, s2 and m2, or s2 and m1. Most H. pylori isolates were of the vacA s1a/m1 type, which has been shown to be associated with advanced diseases based on genotyping of H. pylori from gastric cancer patients. These results demonstrated that H. pylori survives in water and may be a potential source of H. pylori transmission, especially where water is not adequately treated.

Helicobacter pylori, a Gram-negative, microaerophilic bacterium, has been implicated in the etiology of most gastritis and duodenal ulcers and is believed to play a role in gastric cancer (3). Water supplies contaminated with fecal material may be a potential source of H. pylori transmission (13). This is particularly relevant in developing countries where municipal water supplies are not adequately treated and water is obtained from rivers and other untreated sources (9, 15, 19). Although no evidence of association of human infection with water source has been reported in the United States, H. pylori has been detected in sewage, surface water, and shallow groundwater by various methods (11–13; K. Forrest, M. Stinson, and S. M. Wright, Abstr. 98th Gen. Meet. Am. Soc. Microbiol., abstr. Q-48, p. 445, 1998).

To date, there is no standard method for detecting H. pylori in environmental samples. The use of nonstandardized methods complicates comparisons across studies; in addition, the accuracy of results varies according to the sensitivities and specificities of the detection methods employed. Objectives of this research were (i) to isolate H. pylori from untreated wastewater by utilizing a combination of immunomagnetic separation (IMS) and culturing techniques, (ii) to identify H. pylori by classical microbiological tests and PCR (16S rRNA), and (iii) to genotype H. pylori isolates by PCR utilizing the vacuolating cytotoxin A gene (vacA).

Wastewater samples. Raw municipal wastewater was obtained from an open wastewater canal on the U.S.-Mexico border in Ciudad Juárez, Mexico, a city of 1.4 million inhabitants. The study area has an H. pylori prevalence rate of 74% (7, 10, 21). From November to December 1999, several 1-liter water samples were collected from Ciudad Juárez as needed, transported to the lab on ice, and used within 2 h of collection. After isolation techniques were developed, a final 100-ml sample was collected and was the source for all results presented here. The final protocol developed involved starting with an 11-ml aliquot of the water sample, which was analyzed for total fecal coliforms and Escherichia coli by the U.S. Environmental Protection Agency-approved membrane filtration method (2). The H. pylori isolation protocol developed utilized a 1-ml wastewater sample, which was diluted 1:100 with sterile water and concentrated by vacuum filtration on a 2-μm-pore-size nitrocellulose filter, eluted into tryptic soy broth containing antibiotics (5 μg of vancomycin per ml, 0.2 μg of polymyxin per ml, 4 μg of trimethoprim per ml; EM Science, Gibbstown, N.J.), and incubated at 37°C for 24 h in a microaerophilic atmosphere using Bio-Bags (type Cfj; Becton Dickinson). These enriched cultures were harvested and resuspended in phosphate-buffered saline, pH 7.2 (PBS), for IMS. H. pylori control strains 26695, 60190, NCTC 11639, and Tx50a (American Type Culture Collection, Manassas, Va.) were cultured on Columbia agar plates containing 15% defibrinated sheep blood in the microaerophilic atmosphere described above at 37°C for 3 to 5 days.

IMS. An important initial step was a concentration procedure by IMS of untreated wastewater. This step not only selected for H. pylori based on immunological properties but also eliminated contaminating substances that may interfere with culturing. H. pylori immunomagnetic beads were prepared according to the manufacturer’s instructions. Monoclonal mouse anti-H. pylori immunoglobulin G (Fitzgerald Corp., Concord, Mass.) was gently agitated on a rotating tube inverter (Dynal Corp., Oslo, Norway) with 5 ml of magnetic beads precoated with sheep anti-rabbit immunoglobulin G (Dynabeads M-280; Dynal Corp.) for 24 h at 4°C. After being washed, the beads were resuspended in 5 ml of PBS containing 0.1% bovine serum albumin and stored at 4°C (6).

Wastewater samples were mixed with the prepared beads in the proportion of 1 ml to 20 μl and gently agitated as described above for 1 h at 4°C. After being separated and washed in PBS, the bead-H. pylori conjugates were streaked onto Columbia agar-blood agar plates and incubated for 3 to 5 days under microaerophilic conditions at 37°C. Small, gray colonies were...
TABLE 1. Primers used for PCR-based genotyping and sequencing of vacA

<table>
<thead>
<tr>
<th>Primer</th>
<th>Target gene</th>
<th>Nucleotide sequence</th>
<th>Optimal annealing Temp (°C)</th>
<th>Genome positions (expected size [bp])</th>
</tr>
</thead>
<tbody>
<tr>
<td>HP1</td>
<td>16S rRNA</td>
<td>5’GCAATCGGAGCTCGTAGAAAGTTGC3’</td>
<td>55</td>
<td>773–1293 (521)*</td>
</tr>
<tr>
<td>HP2</td>
<td>vacA s1a</td>
<td>5’TCAACCATCAGGCAAGCAGCA3’</td>
<td>55</td>
<td>70–259 (190)*</td>
</tr>
<tr>
<td>S-R</td>
<td>vacA s1b</td>
<td>5’AGCCACATCGGCAAGCAGCA3’</td>
<td>55</td>
<td>73–259 (187)b</td>
</tr>
<tr>
<td>S-R</td>
<td>vacA s2</td>
<td>5’CTGGTCGAGTGGGCAAGCAGCA3’</td>
<td>55</td>
<td>88–286 (199)c</td>
</tr>
<tr>
<td>M1</td>
<td>vacA m1</td>
<td>5’GGTCAAAATGCGGTCATGG3’</td>
<td>50</td>
<td>1954–2243 (290)*</td>
</tr>
<tr>
<td>M1</td>
<td>vacA m2</td>
<td>5’GGAGGCCCAGGAGAACATTG3’</td>
<td>47</td>
<td>1939–2290 (352)*</td>
</tr>
<tr>
<td>M2</td>
<td>vacA m2</td>
<td>5’CACCTAGCCGCTGGC3’</td>
<td>47</td>
<td>1939–2290 (352)*</td>
</tr>
</tbody>
</table>

* H. pylori 26695 (cytotoxic strain with the s1a/m1 vacA type).

b H. pylori 399 (s1b/m1 vacA type).

*c H. pylori Tx30a (nontoxic strain with the s2/m2 vacA type).

selected and stained with Gram stain to verify morphology. Colonies with gram-negative rods or coccolid forms were tested by three diagnostic techniques: a rapid urease test (Christensen’s urea test; Remel Inc., Lenexa, Kans.), cytochrome oxidase test (SpotTest Oxidase kit; Difco, Detroit, Mich.), and catalase test (SpotTest Catalase kit; Difco). H. pylori must be gram negative and positive for all three tests.

**DNA isolation and vacA genotyping.** DNA was extracted from putative H. pylori cells utilizing a prepared kit (Dynabeads DNA DIRECT system I; Dynal Corp.). DNA concentrations were measured by a fluorescence dye assay (DNA quantitation kit; Sigma Chemical Co., St. Louis, Mo.), and samples were diluted to 10 ng/μl for the PCR assay. The method of genotyping H. pylori was derived from previous H. pylori genotyping by Atherton et al. (1). In the Western Hemisphere, the vacA gene has two major allelic variants: s1/s2 and m1/m2. In addition, two minor variants of s1 can be distinguished, s1a and s1b. H. pylori control strains used in this study are known with respect to their vacA genotypes: strains 26695 and 60190 are of the s1a/m2 type, and strain Tx30a is of the vacA s1a/m1 type.

**PCR amplification and DNA sequencing.** Primer sequences for the H. pylori 16S rRNA gene (14) and vacA alleles (1) used in this study are listed in Table 1. PCR amplification was performed according to the following profile: 94°C for 2 min and 40 cycles of 94°C for 45 s, 55°C for 45 s, and 72°C for 1 min, followed by 72°C for 7 min (Robocycler; Stratagene Corp., La Jolla, Calif.). Optimal annealing temperatures were determined by a thermal-gradient program. Some PCR-amplified DNA fragments were cut from the agarose gel after electrophoresis and purified with a concentrator column (GeneClean spin kit; Bio 101, Vista, Calif.).

**Sequence analysis.** Sequencing was performed by both strands by the fluorescent dideoxy terminator method (Sequitherm EXCELII, Epicentre Technologies, Madison, Wis.). Electrophoresis was conducted on an automated DNA sequencer (model 4200; LI-COR), and sequences were analyzed with e-Seq DNA sequencing software 1.0 (LI-COR). Sequences were aligned to the corresponding published sequences of H. pylori American Type Culture Collection strains with the DNA analytical software MacVector 6.5.3. We used previously published vacA open reading frames from the following strains: 26695, 60190, and Tx30a (1, 4, 22).

**H. pylori isolates and analyses.** A total of 37 out of 132 isolates (W1 to W132) were initially selected as putative H. pylori isolates. Selection was based on four standard microbiological tests: Gram staining and urease, oxidase, and catalase tests. Cell morphology was either that of a rod, the shape of a coccus, or the shape of a coccalbacillus. The wastewater sample had approximately 10^5 total coliform and 10^5 E. coli organisms per 100 ml as enumerated by the membrane filtration method. Treated water typically has between <10^3 and 0 E. coli organisms per 100 ml (2).

A total of 37 out of 37 isolates were confirmed to be H. pylori by 16S rRNA PCR. Primers HP1 and HP2 amplified a 520-bp fragment from the H. pylori-positive control strains 26695, 60190, Tx30a, and NCTC 11639 and H. pylori wastewater isolates. As a negative control, three unrelated microorganisms (E. coli, Staphylococcus aureus, and Bacillus cereus) were chosen and tested with the same primers. HP1 and HP2 did not produce any PCR product from the negative controls.

The 16S rRNA-PCR DNA fragments from two randomly selected isolates (W10 and W100) and two control strains (26695 and 60190) were sequenced. The sequences of the fragments from H. pylori strains 26695 and 60190 were nearly identical to that of the published sequence (22). The nucleotide sequence of isolate W10 was identical to that of strain 26695, and that of isolate W100 was 99.8% homologous. The sizes of the PCR-amplified 16S rRNA fragments and confirmatory DNA sequencing indicated that W10 and W100 were true H. pylori isolates.

**vacA alleles.** A set of primers, S1a-F and S-R, were employed to identify the vacA s1a allele. We were able to completely genotype only 11 of the 23 isolates with respect to their vacA genes due to difficulty with primers binding to DNA templates after storage. Of these, eight H. pylori isolates were identified as vacA s1a, and all produced DNA fragments of the expected size, about 190 bp (Table 2). From the control and one wastewater isolate (W10), slu fragments were sequenced for confirmation. The sequence of the s1a fragment from strain 26695 was identical to the published sequence (22). The sequence of
the \textit{s}la fragment from W10 was identical to that of strain 26695.

Primers S1b-F and S-R were used to identify the \textit{vacA} \textit{s}lb allele, and one isolate (W22) was identified with a DNA fragment having the expected size of about 180 bp (Table 2). Five \textit{H. pylori} isolates were identified as having the \textit{vacA} \textit{s}2 allele; however, two isolates, W96 and W116, produced larger PCR-amplified fragments (250 bp) than expected (Table 2). Two isolates, W56 and W116, were also identified as \textit{vacA} \textit{s}la. This finding suggested that isolates W56 and W116 were mixed-genotype colonies or heterogeneous for this allele.

Five isolates were identified as \textit{vacA} \textit{m}1 and had the expected PCR fragment size of 290 bp (Table 2). Four isolates, W41, W53, W56, and W115, produced amplified DNA fragments with sizes larger than 290 bp, ranging from 350 to 450 bp. Their large PCR products may be due to an insertion or nonspecific primer binding, but this was not confirmed by sequencing. Three isolates were identified as \textit{vacA} \textit{m}2 and had the expected PCR-amplified fragment size of about 350 bp. One of these three isolates, W56, contained both \textit{m}1 and \textit{m}2, as had been noted by other researchers (5).

The 16S rRNA PCR assay was shown to be accurate by sequencing of the PCR products of W10 and W100 and comparison of sequences with published 16S rRNA sequences for homology. Other studies (13, 17, 18) have performed PCR directly on gastric biopsy, stool, dental-plaque, or water samples. These studies leave open the question of whether results were inaccurate due to nonspecific primer binding and the presence of interfering substances in the PCR assay. In our research, these potential problems were avoided by isolating and culturing \textit{H. pylori}, isolating DNAs from cultured cells, and then performing PCR on the purified DNAs. Not all isolates passing the initial microbiology tests could be confirmed by \textit{H. pylori} 16S rRNA PCR. Tests on isolates that did not have positive 16S rRNA test results were repeated two times before we concluded that they were negative. The reason why some putative \textit{H. pylori} isolates could not be confirmed by PCR was not further investigated.

\textit{H. pylori} isolates from Ciudad Juárez wastewater demonstrated DNA heterogeneity with respect to the \textit{vacA} gene. Nearly half of the 11 isolates that were fully typed had the \textit{vacA} \textit{s}la/m1 genotype. This allelic combination has been reported to be associated most frequently with ulcer disease (8). It has also been reported that the \textit{s}lb type is more prevalent in Central and South America than in the United States (23). Our finding that the \textit{s}la type is more prevalent may result from our examination of isolates from wastewater, rather than from the stools of patients examined by endoscopy, the source for most other studies. A possible explanation for this difference is that wastewater better captures the distribution of strain types in this combined symptomatic and asymptomatic population. A noteworthy finding was the observation of the allelic type \textit{s}2/m1, which has been reported only recently (16, 20).

Review of the literature has not revealed other published studies that used PCR-based 16S rRNA and \textit{vacA} genotyping of \textit{H. pylori} water isolates. Based on this approach, our study demonstrated the culturability and identity of \textit{H. pylori} in water samples and that this organism can survive several days in water. These findings suggested that with fecally contaminated waters, the potential exists for fecal-oral transmission of \textit{H. pylori} via water.

This work was supported by the Border Biomedical Research Center grant G12–RR08124 from the NIH.

\begin{table}[h!]
\centering
\begin{tabular}{|c|c|c|c|c|c|}
\hline
Isolate & \textit{vacA} fragment size (bp) & \textit{s}la & \textit{s}lb & \textit{s}2 & \textit{m}1 & \textit{m}2 \\
\hline W10 & 190 & - & - & 290 & - & \textit{s}la/m1 \\
W22 & 190 & 187 & - & 290 & - & \textit{s}la and \textit{s}lb/m1 \\
W41 & 190 & - & - & (450) & - & \textit{s}la/m1 \\
W53 & 190 & - & - & (400) & - & \textit{s}la/m1 \\
W56 & 190 & 199 & - & (400) & 352 & \textit{s}la \textit{s}2 \textit{m}1 \textit{m}2 \\
W92 & 190 & - & - & 290 & - & \textit{s}la/m1 \\
W99 & - & - & (250) & - & 352 & \textit{s}2/m2 \\
W100 & - & - & 199 & - & 352 & \textit{s}2/m2 \\
W115 & - & - & (250) & (350) & - & \textit{s}2/m1 \\
W116 & 190 & - & 199 & 290 & - & \textit{s}la and \textit{s}2/m1 \\
W122 & 190 & - & - & 290 & - & \textit{s}la/m1 \\
\hline
\end{tabular}
\caption{\textit{vacA} genotypes of \textit{H. pylori} wastewater isolates}
\end{table}

\begin{flushright}
\textsuperscript{a} PCR-amplified fragment. Values in parentheses indicate sizes different from those expected. —, no PCR-amplified fragment.
\end{flushright}

\textbf{REFERENCES}


