DNA-level characterization of Helicobacter pylori strains from patients with overt disease and with benign infections in Bangladesh

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DNA-Level Characterization of Helicobacter pylori Strains from Patients with Overt Disease and with Benign Infections in Bangladesh

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The complex relation between the genotype of Helicobacter pylori and its association with clinical outcome is not well understood. Studies in the West have showed that strains expressing certain virulence factors (vacA s1, vacA m1, and cagA) are associated with duodenal ulcer disease. However, the H. pylori genotype is known to vary with geographic region. In the present study, we compared several virulence markers (cagA, vacA, and iceA) and neutral markers (IS605, IS606, and IS608) in H. pylori strains isolated from 65 adult patients with peptic ulcer (PU) and 50 patients with nonulcer dyspepsia (NUD). PCR tests indicated that cagA is present in 75% of the strains from patients with PU compared to 55% in patients with NUD, and 80% of the isolates from patients with PU carried potentially toxigenic vacA s1 alleles of the vacuolating cytotoxin gene (vacA) compared to 60% in isolates from patients with NUD. However, no significant difference in any other virulence marker was observed in isolates from both groups. Phylogenetic analysis of the vacA middle region and the 5′ end of the cagA gene indicates that Bangladeshi isolates are more closely related to H. pylori isolates from India and are different from isolates from East Asia.

Helicobacter pylori is a spiral, gram-negative microaerophilic bacterium that chronically infects the gastric mucosa of more than half of all people worldwide (30) and is a major cause of gastritis and peptic ulcer (PU) disease (37), and a risk factor for gastric adenocarcinoma and mucosa-associated lymphoid tissue lymphoma (29). Infection by H. pylori usually starts early in life and, unless eradicated by specific antibiotic treatment, persists for the rest of life. H. pylori produces a number of virulence factors that are essential for colonization of the stomach and survival in the hostile gastric environment (27). Well known virulence factors are urease, which plays an important role in the neutralization of gastric acid secretion (19); flagella, which are essential for swimming through the mucus layer (21); vacuolating cytotoxins, which induce massive vacuolar degeneration of various epithelial cell lines (20); gene products encoded by cag pathogenicity island (PAI), which causes up-regulation of cytokines, including interleukin-8 (2); iceA, a homologue of a gene for restriction endonuclease, induced by contact with gastric epithelium (31); and OipA, a proinflammatory protein that contributes to interleukin-8 induction independent of cag PAI (38).

An intriguing aspect of H. pylori is an extraordinary diversity among strains that is seen in DNA fingerprinting and in tests of gene content and chromosomal gene order (1, 3). Together with this are indications of differences at certain loci between strains from different parts of the world or human ethnic groups. In particular, the DNA sequence motifs and disease association of two virulence genes, vacA and cagA, in strains from the United States and Europe were found to differ from those predominating in southern coastal China and Japan (14, 28, 34, 35), although less phylogenetic clustering was found in sequences of several housekeeping genes (1). However, most of our present understanding of H. pylori genome organization and bacterial traits are based on studies of strains from the West, and it is believed that further studies of H. pylori genotypes from different well-separated human populations may help to increase our understanding of bacterium-host interactions in colonization and disease.

Prevalence studies have indicated that H. pylori infection is extremely common in Bangladesh (22, 32) as in other developing countries. Although the available reports from this country have focused on the clinical patterns of gastroduodenal disease, detection of H. pylori infection, and effectiveness (or ineffectiveness) of anti-H. pylori therapies in the local population (6, 7, 13, 24), none, to our knowledge, provide information on the genotypes of the Bangladeshi H. pylori strains. In this context we have characterized H. pylori strains isolated from Dhaka, the capital of Bangladesh, in order to gain new insights into the population genetic structure of this important human pathogen and to learn if genotypes implicated in the disease in the West are similarly disease associated in Bangladesh.

MATERIALS AND METHODS

Patient samples. Consecutive patients attending the gastroenterology Department of Dhaka Medical College hospital for routine endoscopy were enrolled in
the study. Each subject was informed about the study and written informed consent was obtained under a protocol approved by the Ethical Review Committee of International Centre for Diarrheal Disease Research, Bangladesh. Endoscopy of the subjects was done by using well-washed and disinfected fiber optic endoscopes (model GIF XQ 30; Olympus, Japan). Diagnosis of PU was based on endoscopic examination of the stomach and duodenum during examination. If no evidence of ulcer was found the patient was considered to have nonulcer dyspepsia (NUD) or gastritis, which is a more benign infection. Two biopsy samples were taken from each patient for culture, one from the gastric antrum and one from the corpus. Immediately after collection the biopsy specimen was transported to the laboratory in Stuart transport medium at 4°C.

Bacterial culture. Biopsy samples were vortexed vigorously for 5 min and plated on brain heart infusion agar (Oxoid, Ltd, Basingstoke, Hampshire, United Kingdom) supplemented with 7% sheep blood, 0.4% IsoVitaleX, and Dent supplement (Oxoid). Plates were incubated at 37°C in an atmosphere of 5% O2, 10% CO2, and 85% N2 for 3 to 6 days. 

DNA extraction and PCR. Chromosomal DNA was prepared by the CTAB (hexadecyl-trimethyl ammonium bromide) extraction method (4) from confluent brain heart infusion agar plate cultures. Specific PCR was carried out in a 20-μl volume using 10 ng of DNA, 1 U of Taq polymerase (Promega, Madison, Wis.), 10 pmol of each primer per reaction, 0.25 mM concentration of MgCl2 in standard PCR buffer for 30 cycles using the following conditions: 94°C for 40 s, 55°C for 40 s, and 72°C for a time chosen based on the size of the expected fragment (1 min/kr). PCR primers used for genotyping are listed in Table 1.

DNA sequencing and analysis. The PCR products of the 700-bp middle region of the vacA gene from six strains and 219-bp fragment from the 5’ end of the iceA1 gene from seven strains were purified with a QIAquick gel extraction kit (Qiagen, Valencia, Calif.). DNA sequencing was performed by the dideoxynucleotide chain termination method with an ABI PRISM Dye Termination Cycle Sequencing Ready Reaction kit (Perkin-Elmer-Applied Biosystems, Foster City, Calif.) in an ABI PRISM automated sequencer. DNA sequence editing and analysis were performed with programs in the Genetics Computer Group (Madison, Wis.) package, programs and data in the TIGR H. pylori genome database (http://www.tigr.org/tdb/mdb/hpdb.html), and the PHYLIP program of J. Felsenstein (http://evolution.genetics.washington.edu/phylip.html) (33). Phylogenetic trees were visualized using Treeview (version 1.61; http://www.taxonomy.zoology.gla.ac.uk/rod/rod.html).

RESULTS

Incidence of H. pylori. A total of 115 consecutive patients between 20 and 55 years old were enrolled in the study. Of these 115 patients, 97 (85%) were male and 18 (15%) were female. Among the patients, 65 were diagnosed with PU and 50 were diagnosed with NUD. A total of 57 isolates were obtained from the enrolled subjects. H. pylori was isolated in 37 (56%) of the 65 patients presenting with PU and 20 (40%) of the 50 patients with NUD.

Genotype of H. pylori isolates. (i) vacA gene. The genotype distributions of the strains form patients with PU and NUD is shown in Table 2. The presence or absence of the vacA gene and the empty-site-specific 550-bp ampiclon was scored by PCR with specific primers using DNA extracted from cultured strains (Table 1). A 324-bp fragment indicative of the vacA

<table>
<thead>
<tr>
<th>Region</th>
<th>Primer</th>
<th>Nucleotide sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>vacA1s or vacA2s</td>
<td>VA1-F</td>
<td>5'-ATGGAAATACAAACAAACACAC</td>
</tr>
<tr>
<td>vac m1b</td>
<td>VAm-F</td>
<td>5'-TCGTTGGAATGCGGCAACAC</td>
</tr>
<tr>
<td>vac A2</td>
<td>VAm-R</td>
<td>5'-GGCCCAATGCGATCGATAGT</td>
</tr>
<tr>
<td>vac 0.7-kb middle</td>
<td>VA4-F</td>
<td>5'-GCTTTAGTGCGCTTAAAGAGCAT</td>
</tr>
<tr>
<td>vacA (5' end)</td>
<td>VA4-R</td>
<td>5'-GGAGCCTGAGAAACATTGAT</td>
</tr>
<tr>
<td>cag PAI empty site</td>
<td>Luni 1</td>
<td>5'-CATAAAAGGCCTGAC</td>
</tr>
<tr>
<td>iceA1</td>
<td>iceA1F</td>
<td>5'-GCTTACACCCGATCGTATG</td>
</tr>
<tr>
<td>iceA2</td>
<td>M.Hpy1R</td>
<td>5'-GCTTACACCCGATCGTATG</td>
</tr>
<tr>
<td>iceA1 Δ94 bp</td>
<td>A1F673</td>
<td>5'-GCTTACACCCGATCGTATG</td>
</tr>
<tr>
<td>IS605 orfA</td>
<td>ORF18F</td>
<td>5'-GCTTACACCCGATCGTATG</td>
</tr>
<tr>
<td>IS605 orfB</td>
<td>ORF19F</td>
<td>5'-GCTTACACCCGATCGTATG</td>
</tr>
<tr>
<td>IS606</td>
<td>FB1</td>
<td>5'-GCTTACACCCGATCGTATG</td>
</tr>
<tr>
<td>IS608</td>
<td>608-F1</td>
<td>5'-GCTTACACCCGATCGTATG</td>
</tr>
<tr>
<td>Type I</td>
<td>cagF4856</td>
<td>5'-GCTTACACCCGATCGTATG</td>
</tr>
<tr>
<td>Type II</td>
<td>cagF4856</td>
<td>5'-GCTTACACCCGATCGTATG</td>
</tr>
<tr>
<td>Type III</td>
<td>cagF4856</td>
<td>5'-GCTTACACCCGATCGTATG</td>
</tr>
</tbody>
</table>

The genotype distributions of the strains form patients with PU and NUD is shown in Table 2.
In equivalent PCR tests with DNA from strains of 20 patients with NUD, 12 yielded only the vacAs1 PCR product, 6 yielded the vacAs2 product, and 2 yielded both vacAs1 and vacAs2 products, again indicating mixed infections (Table 2). The general association of cag+ strains and vacAs1 and of strains lacking cag and vacAs2 observed here matches with that seen in many strain collections from the United States and Europe.

The prevalence of the alleles of middle (m) region of vacA, a part that affects host specificity, was also determined by PCR. Products were obtained only with vacAm1b primers in strains of 22 patients with PU and 8 patients with NUD (Table 2; Fig. 1D) whereas PCR amplification with only vacAm2 primers occurred in the cases of 13 of 37 patients with PU and 8 patients with NUD. Both m1b and m2 products were observed in two patients with PU and four patients with NUD.

**Sequence analysis of mid-region alleles of vacA.** DNAs of 0.7-kb PCR fragments amplified with vacA middle region-specific primers containing the vacAm1b region were sequenced for further analysis. The sequence was then compared with available vacAm1a, -m1b, and recently described -m1c sequences in GenBank. Multiple sequence analysis showed all sequences from Bangladeshi isolates are closely related to each other and also closely related (97 to 98% identity) to a recently described vacAm1c allele from Calcutta, India (25). However, only 90 to 91% identity was obtained with both vacAm1b alleles from East Asia and vacAm1a of ethnic European strains. Phylogenetic analysis of the same sequence showed that Bangladeshi isolates are closely clustered to each other and are distantly related to East Asian and ethnic European clusters (Fig. 2B).

(iii) **iceA alleles.** PCR was used to test for iceA1, which is virulence associated in the West, and the completely unrelated iceA2 gene, which can occupy the same chromosomal locus in strains lacking iceA1. The iceA1 gene was in isolates from 19 of 37 patients with PU disease and in nine strains from patients with NUD. In contrast, only the iceA2 gene was present in strains from 17 patients with PU and in 10 strains from patients with NUD. A mixture of iceA1 and iceA2 PCR products, indicating mixed infection, was found in 2 cultures out of a total of 57 (Table 2; Fig. 1E and F). A 94-bp deletion in the iceA1 gene was found in two isolates, both from patients with PU (Fig. 1G).

**cag right junction motifs.** The type III motif at the extreme right end of cag PAI, characterized by the presence of a function-unknown sequence replacing the hel gene fragment (17), was found in 40 of 50 (~80%) cag PAI-positive Bangladeshi strains as determined by PCR with diagnostic primers (Fig. 1H). By PCR, 7 of the remaining 10 strains were found to carry the type I motif, which is common in strains from the United States, Peru, South Africa, and Spain and usually contains a 130-bp remnant of IS606 and about 423 bp of helicase gene fragment (17). One Bangladeshi strain was positive for the type II motif that is predominant in strains of coastal China and Japan and characterized by the presence of 320-bp fragment of IS606 and only a 9-bp remnant of helicase gene (Table 2).

**Distribution of neutral markers.** The prevalence of several mobile DNAs, which should have no effect on colonization or disease, was also studied, since their distribution may be far less affected by selection and thus serve better as a marker of

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**TABLE 2. Genotypic characteristics of H. pylori isolates from Dhaka, Bangladesh, and relation to disease status**

<table>
<thead>
<tr>
<th>Trait or marker*</th>
<th>No. of patients with indicated disease status</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PU (n = 37 [64%])</td>
</tr>
<tr>
<td>cag PAI1 only^b</td>
<td>28</td>
</tr>
<tr>
<td>cag PAI1, cag PAI2 mixed</td>
<td>3</td>
</tr>
<tr>
<td>cag PAI1 only^b</td>
<td>6</td>
</tr>
<tr>
<td>vacA1 only</td>
<td>30</td>
</tr>
<tr>
<td>vacA2</td>
<td>6</td>
</tr>
<tr>
<td>vacAm1, vacA1 mixed</td>
<td>1</td>
</tr>
<tr>
<td>vacAm1c</td>
<td>22</td>
</tr>
<tr>
<td>vacAm2 only</td>
<td>13</td>
</tr>
<tr>
<td>vacAm1c, vacAm2 mixed</td>
<td>2</td>
</tr>
<tr>
<td>iceA1^d</td>
<td>19</td>
</tr>
<tr>
<td>iceA2</td>
<td>17</td>
</tr>
<tr>
<td>iceA1, iceA2 mixed</td>
<td>1</td>
</tr>
<tr>
<td>IS605</td>
<td>16</td>
</tr>
<tr>
<td>IS606</td>
<td>11</td>
</tr>
<tr>
<td>IS608</td>
<td>6</td>
</tr>
<tr>
<td>cag type III^d</td>
<td>26</td>
</tr>
<tr>
<td>cag type II^d</td>
<td>1</td>
</tr>
<tr>
<td>cag type I^d</td>
<td>1</td>
</tr>
</tbody>
</table>

*Distribution of DNA markers was determined by PCR, as illustrated in the figures.

^b cag PAI1 only, infection with strains carrying cag PAI1 only; cag PAI1 - cag PAI2 mixed infection with strains carrying the cag PAI1 and strains lacking the cag PAI1; cag PAI1 only, infection with strains lacking the cag PAI only.

^d A 94-bp deletion was found in two isolates, both from patients with PU.

^c The 3' end of the cag gene was typed and scored as type I, II, or III. Isolates from European origin usually belong to type I.

---

Gene (Fig. 1A) was obtained with primers specific for the cagA gene from the great majority of strains: 28 of 37 cultures from patients with PU and 11 of 20 patients with NUD. Eleven of 18 cultures from which no cagA gene-specific PCR product was obtained yielded an empty-site product of the expected size, 550 bp (Fig. 1B), indicating that they truly lacked the cagA gene (4). In addition, products corresponding to the empty- site product were obtained only with primers containing the vacAm1b region were sequenced for further analysis. The sequence was then compared with available vacAm1a, -m1b, and recently described -m1c sequences in GenBank. Multiple sequence analysis showed all sequences from Bangladeshi isolates are closely related to each other and also closely related (97 to 98% identity) to a recently described vacAm1c allele from Calcutta, India (25). However, only 90 to 91% identity was obtained with both vacAm1b alleles from East Asia and vacAm1a of ethnic European strains. Phylogenetic analysis of the same sequence showed that Bangladeshi isolates are closely clustered to each other and are distantly related to East Asian and ethnic European clusters (Fig. 2B).

**Phylogenetic relationship between cagA 5’ end of Bangladeshi isolates and those from other geographic regions.** To assess the phylogenetic relationship between cag PAIs of Bangladeshi isolates and those from other regions, we sequenced a 219-bp fragment near the 5’ end of the cagA gene that had been used to distinguish east Asian and U.S. and European strains. The sequences were closely related to each other and also to those from Western strains, but not to those from Chinese and Japanese strains (Fig. 2A).

(ii) **vacA alleles.** The presence of the toxigenic vacAs1 or the nontoxigenic vacAs2 allele at the 5’ end of vacA was determined on the basis of the sizes of PCR products generated with specific primers. Of the 37 cultures tested from patients with PU, 30 yielded only a 259-bp fragment, indicating the s1 allele; 6 yielded only a 286-bp fragment, indicating the s2 allele; and the remaining 1 yielded both the 259- and 286-bp fragments, indicating an s1 and s2 mixed infection (Table 2; Fig. 1C).
ancient phylogenetic lineages than alleles of virulence-associated genes. PCR tests identified sequences from IS606 in 18 strains and IS605 in 23 strains of the 57 studied (Table 2). Further, eight cultures contained both these elements, and 23 strains lacked both of them. IS605 was present in 43% (17 of 39), 42% (3 of 7), and 27% (3 of 11) of the cagA gene-positive cultures, mixed cultures, and cultures lacking cagA, respectively. Thus, each element appears to be inherited independently of the others. IS608 was identified in 6 of the 37 isolates from patients with PU and 3 of the 20 patients with NUD.

**DISCUSSION**

*H. pylori* strains from Bangladesh were studied to gain new insight into the population genetic structure of the pathogen and to study the implication of genotype in disease. Although *H. pylori* infection occurs worldwide, there are significant differences in its prevalence both within and between countries (11, 23). In developing countries, including Bangladesh, the majority of children usually get infected during their first 1 to 2 years of life, and most others acquire *H. pylori* even before adulthood (18). An age-related prevalence study in Bangladesh showed that 61% of infants (1 to 3 months old) were positive for *H. pylori*, and the figure rose to 84% in 6- to 9-year-olds (22). Otherwise, the reported seroprevalence of *H. pylori* in the hospitalized Bangladeshi population was 77.4% (26). A high association of *H. pylori* with PU (duodenal ulcer, 77%; gastric ulcer, 75%) and gastritis (74%) was observed in a previous study (12). However, in the present study, *H. pylori* could be cultured from 56% of PU and 40% of patients with NUD reporting at Dhaka Medical College Hospital, Bangladesh. This may be due to a difference in methods for detection of *H. pylori* (other studies used serology, rapid-urease test or histology) and uneven distribution of the organism throughout the gastric mucosa. Since the main focus of this study was to determine the genetic makeup in relation to virulence of Bangladeshi *H. pylori* strains and since culture of *H. pylori* from gastric biopsy specimens undoubtedly constitutes the most specific way to establish the presence of the bacteria and to study the genotype, it was not deemed necessary to employ all other methods for detection of *H. pylori* in the study.

*H. pylori* strains carrying the cag PAI and the potentially toxigenic s1 alleles of the vacuolating cytotoxin (vacA) gene were found to predominate in Bangladesh, but at the same time, some strains carrying the vacAs2 allele and lacking the
FIG. 2. Phylogenetic trees of sequences within the cagA gene and vacA m1 alleles. Sequences from non-Bangladeshi strains that were used here were from public databases, as indicated. (A) Phylogenetic tree based on informative 219-bp segment of cagA of H. pylori strains determined in this study. The tree was generated by using PHYLIP (Phylogeny Inference Package), version 3.5c, of J. Felsenstein. The strains used were as follows (GenBank accession number in parentheses): 1, CHINAR48 (AJ252983); 2, JAPANF32 (AJ239726); 3, JAPANGC4 (AF198484); 4, CHINAR29 (AJ252980); 5, CHINAR59 (AJ252986); 6, CHINAR47 (AJ252985); 7, HK77 (AF198485); 8, TH88-28 (AJ239722); 9, CHINAR29 (AJ252980); 10, HK97-42 (AJ239726); 11, CHINAR60 (AJ252982); 12, HK81 (AF198486); 13, INDIA19 (AF198484); 14, I-9A (AF202225); 15, PERU35B (AF202221); 16, DH140 (AY169293); 17, DH37 (AY169296); 18, India18 (AF202224); 19, DH60 (AY169297); 20, India19 (AF202225); 21, DH29 (AY169295); 22, DH200 (AY169294); 23, DUT79 (AJ252970); 24, DUT25 (AJ252968); 26, Guatemalan88 (AF198472); 27, Gambia4797 (AF198472); 28, South Africa19 (AF198470); 29, Peru4A (AF198477); 30, Gambia4659 (AF198468); 31, Dutch107 (AJ252963); 32, 26695 (AE000569); 33, G31 (AY169299); 34, DH102 (AY169292); 35, Peru34B (AF198475). Isolates from Bangladesh are shown in boldface type. (B) Phylogenetic trees of sequences within the vacA m1 alleles. Sequences from non-Bangladeshi strains used here were from public databases as indicated. The tree was generated by using PHYLIP (Phylogeny Inference Package), version 3.5c, of J. Felsenstein. The
sequences of East Asian, European, and Indian vacA
alleles were taken from GenBank (GenBank accession number in parentheses): 1, GER19 (AF006967); 2, DH131 (AY16928); 3, DH92 (AY16929); 4, India48 (AF20112); 5, DH60 (AY169288); 6, India89 (AF20114); 7, India19 (AF20111); 8, India226 (AF20115); 9, DH153 (AY16926); 10, DH9 (DH169290); 11, DH114 (AY169291); 12, India18 (AF20110); 13, India227 (AF20116); 14, India66 (AF20113); 15, India230 (AF20117); 16, 26695 (AE000598); 17, NCTC11638 (U07145); 18, Poland492 (AF097570); 19, NCTC11637 (AF049653); 20, Poland278 (AF097571); 21, Japan99 (AE001511); 22, Kenya AFN4847 (AF191644); 23, Chile-CH1 (AF479031); 24 Mex467 (AF199855); 25, JapanF63 (AF049635); 26, China13 (AF035610); 27, China39 (AF035611); 28, JapanF52 (AF049631); 29, JapanF55 (AF049632); 30, JapanF72 (AF049651); 31, JapanF61 (AF049652); 32, JapanF42 (AF049626); 33, JapanF36 (AF049624); 34, JapanF64 (AF049674); 35, JapanF35 (AF049625); 36, JapanF45 (AF049628); 37, JapanF64 (AF049629); 38, JapanF57 (AF049634). Isolates from Bangladesh are shown in boldface type.
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REFERENCES

ERRATUM

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