cagA-Positive *Helicobacter pylori* Populations in China and The Netherlands Are Distinct

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Received 26 November 1997/Returned for modification 23 January 1998/Accepted 11 February 1998

The aim of this research was to study whether and to what extent Chinese cagA-positive *Helicobacter pylori* isolates differ from those in The Netherlands. Analysis of random amplified polymorphic DNA (RAPD)-PCR-assessed DNA fingerprints of chromosomal DNA of 24 cagA-positive *H. pylori* isolates from Dutch (n = 12) and Chinese (n = 10) patients yielded the absence of clustering. Based on comparison of the sequence of a 243-nucleotide part of cagA, the Dutch (group I) and Chinese (group II) *H. pylori* isolates formed two separate branches with high confidence limits in the phylogenetic tree. These two clusters were not observed when the sequence of a 240-bp part of glmM was used in the comparison. The number of nonsynonymous substitutions was much higher in cagA than in glmM, indicating positive selection. The average levels of divergence of cagA at the nucleotide and protein levels between group I and II isolates were found to be high, 13.3 and 17.9%, respectively. Possibly, the pathogenicity island (PAI) that has been integrated into the chromosome of the ancestor of *H. pylori* now circulating in China contained a different cagA than the PAI that has been integrated into the chromosome of the ancestor of *H. pylori* now circulating in The Netherlands. We conclude that in China and The Netherlands, two distinct cagA-positive *H. pylori* populations are circulating.

*Helicobacter pylori* infection in humans is one of the most widespread infections today, and its cure prevents peptic ulcer recurrence (26, 35). Besides asymptomatic gastritis and peptic ulcer disease (PUD), *H. pylori* infection is strongly associated with gastric cancer, gastric mucosa-associated lymphoid tissue (MALT), and adenocarcinoma of the stomach (3, 9, 24).

The heterogeneity of the clinical outcome of *H. pylori* infection may be related either to differences among the hosts or to differences in virulence among *H. pylori* strains. The latter assumption is supported by the finding that the product of cytotoxin-associated gene A (cagA) has been found to be associated with PUD (7). PUD patients are virtually all infected with cagA-positive *H. pylori* and have serum antibodies as well as antibodies at the mucosal level against a 120- to 128-kDa protein encoded by this gene (7, 38). In contrast, only 60% of the patients with functional dyspepsia (FD) are positive for this protein. The presence of cagA-positive *H. pylori* is also related to an increased risk to develop atrophic gastritis, intestinal metaplasia (16, 35), or gastric cancer (25).

Recently, the complete genome sequence of *H. pylori* has become available (30). A 40-kb region of the *H. pylori* chromosome containing cagA was sequenced earlier by Censini et al. (4). This locus, comprising at least 40 genes, has a GC content different from that of the rest of the chromosome, forms a so-called pathogenicity island (PAI), and is assumed to have been integrated into the *H. pylori* chromosome only recently (4, 6). The proteins encoded by the PAI genes possess features similar to those of bacterial type II, type III, and most notably type IV secretion systems. It was hypothesized that such proteins may function to export macromolecules that may be involved in the *H. pylori*-host cell interaction (6).

China is one of the countries with a high prevalence of *H. pylori* infection and a high incidence of gastroduodenal diseases (39). The prevalence of *H. pylori* infection increases with age to about 70% of the people over 30 years old (22, 33, 39). The prevalence of cagA-positive *H. pylori* populations in Chinese patients with PUD and FD is almost universally high (21). Data obtained from this recent study further suggested that *H. pylori* genotypes distinct from those present in Western Europe may circulate in China.

The aim of this study is to investigate this hypothesis by comparison of the random amplified polymorphic DNA (RAPD)-PCR-assessed genotype of 24 randomly collected cagA-positive *H. pylori* isolates from 12 Dutch (14 isolates) and 10 Chinese patients. We used four different primers in each of four amplifications of *H. pylori* genomic DNA. In addition, part of cagA and glmM of the *H. pylori* isolates was sequenced. Sequences were analyzed for similarity by a computer-based program by using the neighbor-joining algorithm of Saitou and Nei (27).

**MATERIALS AND METHODS**

Patients and *H. pylori* isolates. In this study, 24 cagA-positive *H. pylori* isolates, 14 from 12 Dutch patients (5 with PUD and 7 with FD) and 10 from 10 Chinese patients (5 with PUD and 5 with FD), were used. Isolates were randomly collected from the collection present in the Department of Medical Microbiology, Academic Medical Center, Amsterdam, The Netherlands. From two Dutch patients, two *H. pylori* isolates were analyzed. These isolates were cultured from biopsy specimens taken with 6-year isolates (isolates 79A and 79I) and 4-year isolates (isolates 161A and 161L) time intervals, respectively. Culture of the *H. pylori* isolates and assessment for the presence of cagA by PCR and Western blotting were recently described (21, 38).

Preparation of genomic DNA for PCR. The chromosomal DNA of *H. pylori* was prepared as previously described (32). Briefly, stored bacterial suspensions were thawed, inoculated on horse blood agar plates, and cultured at 37°C for 3 days in a microaerobic environment. Bacteria were harvested, and genomic DNA
was extracted by phenol-chloroform-isomyl alcohol extraction and ethanol precipitation (32).

**Genome typing by RAPD-PCR.** PCR-based RAPD fingerprinting was performed by the method of Akopyants et al. (1), with minor modifications (32). Briefly, 20 ng of chromosomal DNA and 5 pmol of one of the primers (Perkin-Elmer Nederland BV, Gouda, The Netherlands) 1254 (5'-AACTGAAACTTACTAGGCTG-3'), 1281 (5'-AAAGCGCAGCCAC-3'), 1283 (5'-GCGATCCCCAC-3'), and 1247 (5'-AAAGCCCCGTG-3') (1) were used in a PCR as previously described (32). The PCR fragments were analyzed by horizontal agarose (1%) gel electrophoresis as described before (32).

**Computer-assisted analysis of RAPD patterns.** The RAPD patterns were visualized by UV illumination and imaged with a video camera. Cluster analysis was performed with Gelcomparator software version 3.1 (Applied Biosystems). Patterns were normalized to RAPD patterns from Neisseria meningitidis ET present every five lanes on each gel. The patterns generated by each of the four RAPD primers were compared and used by comparing unweighted pair group method for arithmetic averages (UPGMA) clustering with Dice coefficient applied.

**Fluorescence-based DNA sequencing and analysis.** PCR products obtained with primer cagA45 (5'-GGACATGTTGCTGCGAGGAG-3'), positions 1409 to 1519, according to Covacci et al. (5) and primer cagA42 (5'-GGATATTTTATTCT-3') positions 1819 to 1927 (21) were subjected to a PCR-based sequencing in both directions by reaction with fluorescent dye-labeled dideoxynucleotide terminators, using the Taq polymerase (Perkin-Elmer) and primer sequences were compared by using a computer program DNASIS to analyze possible recombination events in the sequenced part of the cagA.

**RESULTS**

**RAPD-PCR of H. pylori isolates from Dutch and Chinese patients.** Assessment by RAPD-PCR of chromosomal DNA of 22 cagA-positive H. pylori isolates, 12 from 12 Dutch patients and 10 from 10 Chinese patients, showed that each isolate had a unique RAPD pattern. The initial isolate 79A and isolate 79J cultured from sequential biopsy specimens taken from the same patient were identical. Likewise, the initial isolate 161A was identical to isolate 161L. Clustering analysis did not reveal any clusters of isolates on the basis of either clinical manifestations or origin of geographic area.

**Comparison of cagA sequences of H. pylori isolates from Dutch and Chinese patients.** Comparison of the 243-bp part of the cagA sequence region between nucleotides 1537 and 1780 (notation according to Covacci et al. [5]) from the 24 clinical H. pylori isolates showed 21 alleles, with mutations at 67 possible positions (Fig. 1). Both sequentially recovered H. pylori isolates from two Dutch patients (strains 161A and 161L; strains 79A and 79J) and two H. pylori isolates from two Chinese patients (strain R27 and R30) had identical cagA sequences. In Fig. 2, the polymorphic site in the cagA region between nucleotides 1537 and 1780 of cagA is shown. The total number of 67 nucleotide substitutions resulted in 22 possible amino acid substitutions. The $d_s$ and $d_v$ values were similar in the 12 H. pylori isolates from 12 Dutch patients and the group of H. pylori isolates from 10 Chinese patients (Table 1).

Clustering analysis revealed two major groups comprising the H. pylori strains from all Dutch patients (group I) and the H. pylori strains from all Chinese patients (group II) (Fig. 2). Bootstrap analysis (1,000 replicates) demonstrated a high confidence (that is, identical branch points occurred in all bootstrap replicates) of the difference between the two main groups comprising the H. pylori isolates from Dutch and Chinese patients. The cagA sequence of group I strains (excluding strains 79J and 161L) showed 3.9% average divergence at the nucleotide level and 6.2% average divergence at the amino acid level.
level. The levels of average divergence of the cagA sequence among the group II strains were similar, 4.8 and 5.8% at the nucleotide and amino acid levels, respectively. Evidently, the difference in the cagA sequence was more extensive (two to three times larger) when the strains of the two groups were compared with each other (Table 2).

Comparison of glmM sequences of H. pylori isolates from Dutch and Chinese patients. To compare sequence heterogeneity of cagA, located on the PAI, with that of a gene outside the PAI, part of glmM (formerly called ureC [18]) was sequenced. Of the 24 H. pylori isolates, the same 240-bp part of glmM was sequenced as described by Kansau et al. (14). Twenty-two alleles with mutations at 32 possible positions were found (Fig. 3). The two sequentially recovered H. pylori isolates from each of the two Dutch patients (strains 161A and 161L; strains 79A and 79J) were identical. The total number of 32 nucleotide substitutions resulted in only 3 possible amino acid substitutions. The $d_s/d_N$ ratio ($d_s/d_N = 0.1103/0.0068 = 16.2$) was much higher in glmM than in cagA. In contrast to the cagA sequence, clustering analysis of glmM did not result in any robust cluster formation.

**DISCUSSION**

Data obtained from a recent report suggested that H. pylori genotypes circulating in China are distinct from those in Western Europe due to allelic variation in cagA (21). The aim of our study was to provide evidence that Chinese patients and Dutch patients are colonized with distinct cagA-positive H. pylori strains.

RAPD-PCR analysis of 14 H. pylori isolates from 12 Dutch patients and 10 from 10 Chinese patients demonstrated a high level of genetic diversity among the 24 strains. In previous studies using this technique, it was shown that H. pylori comprises a genetically highly heterogeneous group, with patient-to-patient variation (1). In addition, patients can harbor a heterogeneous H. pylori population (12, 31, 33, 37). On the basis of the RAPD-PCR patterns, the 24 H. pylori strains could be clustered according to neither the various clinical entities nor the geographic origin of the patient. Results obtained with multilocus enzyme electrophoresis suggested clustering of 23 H. pylori isolates into four clusters (11). The authors concluded that the genetic diversity in H. pylori may be sufficient to classify H. pylori strains into four or more cryptic species.

**TABLE 1.** Proportion (Jukes-Cantor corrected) of synonymous and nonsynonymous substitutions per site among the 243-nucleotide sequenced part of cagA between nucleotides 1573 and 1780 (notation according to Covacci et al. [5]) of 25 H. pylori isolates

<table>
<thead>
<tr>
<th>Source of isolates</th>
<th>No. of isolates</th>
<th>Proportion of substitutions (mean ± SE)</th>
<th>$d_s/d_N$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dutch patients</td>
<td>12</td>
<td>$0.102 ± 0.071$</td>
<td>0.027 ± 0.021</td>
</tr>
<tr>
<td>Chinese patients</td>
<td>10</td>
<td>$0.140 ± 0.053$</td>
<td>0.025 ± 0.010</td>
</tr>
</tbody>
</table>

The cagA sequences of H. pylori isolate 79J (identical to 79A but isolated from the same patient 6 years later) and 161L (identical to 161A but isolated from the same patient 4 years later) were not taken into account.

**TABLE 2.** Sequence diversity among a part of 243 nucleotides of the cagA region between 1573 and 1780 (notation according to Covacci et al. [5]) of H. pylori isolates from 12 Dutch and 10 Chinese patients

<table>
<thead>
<tr>
<th>Source of isolates</th>
<th>No. of isolates</th>
<th>% Differences (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Nucleotide</td>
</tr>
<tr>
<td>Group I</td>
<td>12</td>
<td>3.9 ± 2.5</td>
</tr>
<tr>
<td>Group II</td>
<td>10</td>
<td>4.8 ± 1.6</td>
</tr>
<tr>
<td>Groups I and II</td>
<td>22</td>
<td>13.3 ± 1.4</td>
</tr>
</tbody>
</table>

$^a$ Group I, Dutch patients; group II, Chinese patients.

$^b$ The cagA sequences of H. pylori isolate 79J (identical to 79A but isolated from the same patient 6 years later) and 161L (identical to 161A but isolated from the same patient 4 years later) were not taken into account.
shown. The numbers (in vertical format) above the sequences identify positions or more differences between cagA. The evolved due to genetic differences of the hosts or other environmental conditions. The evolved in China contained a different difference between H. pylori altering the robust division between the Western and Chinese populations are circulating in China and The Netherlands. Most likely, the PAI that has been integrated into the chromosome of the ancestor of the H. pylori populations are circulating in China and The Netherlands. Most likely, the PAI that has been integrated into the chromosome of the ancestor of the H. pylori now circulating in China contained a different cagA than the PAI that has been integrated into the chromosome of the ancestor of the H. pylori now circulating in The Netherlands. Presented here is the conclusion that two distinct cagA-positive H. pylori populations are circulating in China and The Netherlands. Most likely, the PAI that has been integrated into the chromosome of the ancestor of the H. pylori now circulating in China contained a different cagA than the PAI that has been integrated into the chromosome of the ancestor of the H. pylori now circulating in The Netherlands.

**ACKNOWLEDGMENTS**

This work was supported by grants from the Dutch Ministry of Education and Science, the Royal Dutch Academy of Science, and the Chinese Ministry of Public Health (1994).

**REFERENCES**

AUTHORS’ CORRECTIONS

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Volume 66, no. 5, p. 1822–1826, 1998. Page 1823, column 2: The following paragraph should be inserted at the end of Materials and Methods:

**Nucleotide sequence accession numbers.** The nucleotide sequences of *cagA* and *glmM* have been deposited in the GenBank database under accession no. AJ252963 to AJ252986 and AJ252987 to AJ253010, respectively.

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Genomic Analysis Reveals Variation between *Mycobacterium tuberculosis* H37Rv and the Attenuated *M. tuberculosis* H37Ra Strain

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Volume 67, no. 11, p. 5768–5774, 1999. Page 5773, column 2, “Acknowledgments”: Because of an administrative error, the second paragraph was incomplete and should read as follows:

“Financial support for this work was provided by the Wellcome Trust, the Biomed Program of the European Community (grant BMH4/CT97/2277), the Institut Pasteur, and l’Association Française Raoul Follereau. S. V. Gordon was the recipient of a Wellcome Trust International Travelling fellowship.”