Assessment of East Asian-type cagA-positive *Helicobacter pylori* using stool specimens from asymptomatic healthy Japanese individuals

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Recent investigations have suggested that CagA, a virulence factor of *Helicobacter pylori* and known to have multiple genotypes, plays a critical role in the development of stomach cancer. However, the prevalence of cagA-positive *H. pylori* strains and the cagA genotypes have not been well studied in healthy individuals because of the difficulty in collecting gastric specimens. In the present study, we assessed the prevalence of infection with *H. pylori*, particularly the strains with the East Asian cagA genotype (which is more potent in causing gastric diseases), among healthy asymptomatic Japanese individuals by a noninvasive method using stool specimens. The *H. pylori* antigen was detected in 40.3 % of healthy asymptomatic adult individuals (n = 186) enrolled in the study. For the detection and genotyping of the cagA gene, DNA was extracted from the stool specimens of these individuals and analysed by PCR. We detected the East Asian cagA genotype in the DNA samples of a significantly high number (63.1 %) of healthy asymptomatic Japanese individuals. These results indicate that a significant number of asymptomatic healthy Japanese individuals were infected with highly virulent *H. pylori*.

**INTRODUCTION**

The significance of various virulence factors of *Helicobacter pylori* has been studied with regard to the pathology of gastric diseases, such as peptic ulcers and gastric cancer. Among these, CagA is one of the well-studied virulence factors of *H. pylori*. The CagA protein is classified into two major types – the East Asian type and the Western type – depending on the combination of the four domains (A, B, C and D) present on the variable region of the C-terminal domain of this protein (Higashi *et al.*, 2002a; Yamaoka *et al.*, 2000b). Each domain contains a single Glu-Pro-Ile-Tyr-Ala (EPIYA) motif (Covacci *et al.*, 1993). Recent studies have revealed that EPIYA motifs are potential targets of the Src family of protein tyrosine kinases. Furthermore, of the four EPIYA motifs in each domain, EPIYA-D of the East Asian CagA protein has a stronger transforming activity than that of the Western type because of the stimulation of signal transduction cascades (Higashi *et al.*, 2002b; Naito *et al.*, 2006). Therefore, the East Asian CagA is more virulent than the Western type, which contains the EPIYA-C domain. In addition, it has been reported that the East Asian CagA probably plays a more effective pathophysiological role than the Western type in the development of gastric diseases caused by *H. pylori* infection (Azuma, 2004). Therefore, extensive genotyping of the cagA gene has been carried out in many countries using *H. pylori* strains clinically isolated from patients with gastric diseases (Devi *et al.*, 2006; Kanata *et al.*, 2008; Vilaichone *et al.*, 2004; Yamaoka *et al.*, 2000b, 2008; Yamazaki *et al.*, 2005b).

In contrast, there have been only few studies on the different cagA genotypes of *H. pylori* in healthy asymptomatic individuals, mainly because of the difficulty in collecting gastric biopsy samples from healthy individuals (Chattopadhyay *et al.*, 2002; Molnar *et al.*, 2008; Yamaoka *et al.*, 2000a). Therefore, we established a genotyping method that involves the use of stool specimens, which were collected from healthy asymptomatic individuals (Hirai *et al.*, 2009). In the present study, we determined the incidence of *H. pylori* infection by using a noninvasive method to analyse stool specimens and detected the East Asian cagA genotype in healthy asymptomatic Japanese individuals.

**METHODS**

**Participants and stool specimens.** This study was conducted in Osaka, Japan, from June 2007 to October 2007. Initially, a total of 235 individuals were enrolled in this study. These individuals were screened for age (>39 years) and medical history. The exclusion
criteria included any antibiotic treatment in the past 3 months, eradication therapy for _H. pylori_, and a confirmed diagnosis of digestive tract diseases. Finally, 186 individuals (65 women, 121 men; age range 40–63 years) participated in this study. Stool specimens were collected from the participants, and they were also asked to fill out questionnaires. This study was approved by the ethics committee of the Osaka University Graduate School of Medicine, Osaka, Japan.

**Detection of the _H. pylori_ antigen and DNA extraction.** The individuals’ stool specimens were tested for catalase, i.e., an _H. pylori_ antigen, by immunochromatographic analysis using a commercially available rapid test kit (TestMate Rapid _Pylori_ Antigen; BD Japan), according to the manufacturer’s instructions (Cardenas _et al._, 2008; Suzuki _et al._, 2002). The detection limit of this kit is 18.8 ng ml⁻¹ of protein concentration (equivalent to 10⁵–10⁶ bacterial cells ml⁻¹). Bacterial DNA was extracted from stool specimens that tested positive for the _H. pylori_ antigen using the QIAamp DNA stool mini kit (Qiagen) according to the manufacturer’s instructions with the following minor modifications. Approximately 1 g of each stool specimen was suspended in 3.0 ml ASL buffer (supplied in the kit). After mixing the suspension, approximately 1.2 ml of the supernatant was used for DNA extraction. The extracted DNA was dissolved in 200 μl AE buffer (supplied in the kit) and stored at −20 °C until further use.

**PCR analysis.** All PCR analyses were performed according to methods described in a previous report (Hirai _et al._, 2009). The sequences of the primers and the probe used in this study are shown in Table 1. For the template, we used a solution containing 50 ng DNA μl⁻¹ or a 10-fold dilution of the DNA sample (approx. 5–10 ng μl⁻¹), which minimized the effect of the inhibitors possibly present in the solution. We performed real-time PCR to detect the 16S rRNA gene of _H. pylori_. In order to detect the East Asian _cagA_ genotype, nested PCR was performed using genotype-specific primers. We performed two rounds of PCR. The first round was performed using a common forward primer (F1) and either of the two reverse primers (R1 or R2) (Fig. 1b, c). The PCR cycling conditions for the first round were as follows: 95 °C for 10 min, then 40 cycles at 94 °C for 15 s, 55 °C for 30 s and 68 °C for 30 s. The second round was performed using 1 μl of the PCR products obtained in the first round as the template. In the second round, primers specific to these two types were used in separate reactions. The cycling conditions of the second round of PCR were as follows: 94 °C for 2 min, then 30 cycles at 98 °C for 10 s and 63 °C for 30 s. The PCR products were electrophoresed on a 2% agarose gel and visualized by ethidium bromide staining.

**Data analysis.** All results were analysed by χ² analysis. The level of statistical significance was set at _P_ <0.05.

### Table 1. Oligonucleotide primers and a probe used for PCR analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer/probe</th>
<th>Sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S rRNA</td>
<td>Forward</td>
<td>5’-TGC GAA GTG GAT GCA ATC TT-3’</td>
<td>Yamazaki <em>et al.</em> (2005a)</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’-GGA ACG TAT TCA CCG CAA CA-3’</td>
<td>Yamazaki <em>et al.</em> (2005a)</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>5’-(FAM) CCT CTC AGT TCG GAT TGT AGG CTG CAA C (TAMRA)-3’</td>
<td>Yamazaki <em>et al.</em> (2005a)</td>
</tr>
<tr>
<td><em>cagA</em></td>
<td>F1</td>
<td>5’-GGA ACC CTA GTC AGT AAT GGG TT-3’</td>
<td>Hirai <em>et al.</em> (2009)</td>
</tr>
<tr>
<td></td>
<td>F2</td>
<td>5’-CCA ATA ACA ATA ATG GAC TCA A-3’</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>R1</td>
<td>5’-GCT TTA GCT TCT GAT ACC GCT TGA-3’</td>
<td>Hirai <em>et al.</em> (2009)</td>
</tr>
<tr>
<td></td>
<td>R2</td>
<td>5’-AAT TCT TGT TCC CTT GAA AGC CC-3’</td>
<td>Hirai <em>et al.</em> (2009)</td>
</tr>
<tr>
<td></td>
<td>EA-F</td>
<td>5’-AAA GGA GTG GGC GGT TTC TCA A-3’</td>
<td>Yamazaki <em>et al.</em> (2005a)</td>
</tr>
<tr>
<td></td>
<td>EA-R</td>
<td>5’-CCT GCT TGA TTT GCC TCA TCA-3’</td>
<td>Yamazaki <em>et al.</em> (2005a)</td>
</tr>
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</table>

### RESULTS AND DISCUSSION

#### Prevalence of _H. pylori_

In Japan, there has been a considerable increase in the incidence of gastric cancer in middle-aged individuals (Yoshida _et al._, 2006); hence, for this study, middle-aged and older individuals who did not exhibit any subjective symptoms and had not received medical treatment in the previous 3 months were enrolled. The age distribution of the 186 participants enrolled in this study is shown in Table 2. Of the 186 participants, 75 (40.3%) tested positive for the _H. pylori_ antigen (Table 3). The incidence of _H. pylori_ infection in each age group ranged from 33.3 to 51.2%; no significant difference was observed among the age groups in this regard (Table 2).

The study revealed that a certain number of healthy asymptomatic Japanese individuals who were older than 39 years were infected with _H. pylori_; this was directly determined using the individuals’ stool specimens. However, previous studies that employed the serological method reported higher incidences (70–80%) of _H. pylori_ infection among individuals who were >40 years old (Asaka _et al._, 1992; Fujisawa _et al._, 1999; Yamagata _et al._, 2000). It is likely that the difference between the present results and those reported by previous studies may be attributable to the different detection systems employed in these studies. The serological method may tend to yield a relatively higher infection rate than the antigen detection method using stool specimens because the serological method also detects past and cured infections. It has been shown that the sensitivity and specificity of the antigen detection method used in this study are comparable to those of the breath test, which is widely used as a standard method for the detection of _H. pylori_ infection (Cardenas _et al._, 2008).

#### Detection of the _H. pylori_ 16S rRNA gene

Total bacterial genomic DNA was extracted from stool specimens that tested positive for the _H. pylori_ antigen. In order to confirm the existence of _H. pylori_ genomic DNA in the extracted DNA, real-time PCR was performed using...
primers targeting the 16S rRNA gene (Yamazaki et al., 2005a). The detection limit for the 16S rRNA gene was 10 copies mg⁻¹. As shown in Table 3, the 16S rRNA gene was detected in 65 of 75 (86.7 %) DNA samples obtained from stool samples that tested positive for the H. pylori antigen. This high detection rate indicated that the method used for DNA extraction was effective. In addition, H. pylori DNA was detected in fewer than 5 % of the DNA samples extracted from randomly selected stool specimens that tested negative for the H. pylori antigen. These findings indicate that the results of the present study revealed the prevalence of H. pylori infection in all the study participants.

Table 2. Prevalence of H. pylori infection and the ratio of the East Asian cagA genotype in the age groups

<table>
<thead>
<tr>
<th>Age group</th>
<th>No. tested</th>
<th>No. antigen positive (%)</th>
<th>No. East Asian genotype positive (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>40–45</td>
<td>55</td>
<td>20 (36.4)</td>
<td>8 (14.5)</td>
</tr>
<tr>
<td>46–50</td>
<td>42</td>
<td>16 (38.1)</td>
<td>11 (26.2)</td>
</tr>
<tr>
<td>51–55</td>
<td>42</td>
<td>16 (38.1)</td>
<td>7 (16.7)</td>
</tr>
<tr>
<td>56–60</td>
<td>41</td>
<td>21 (51.2)</td>
<td>13 (31.7)</td>
</tr>
<tr>
<td>&gt;60</td>
<td>6</td>
<td>2 (33.3)</td>
<td>2 (33.3)</td>
</tr>
</tbody>
</table>

Table 3. Summary of H. pylori antigen, DNA and cagA detection

<table>
<thead>
<tr>
<th></th>
<th>No. tested</th>
<th>No. positive (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H. pylori antigen test</td>
<td>186</td>
<td>75 (40.3)</td>
</tr>
<tr>
<td>Real-time PCR 16S rRNA</td>
<td>75</td>
<td>65 (86.7)</td>
</tr>
<tr>
<td>Genotyping of cagA East Asian genotype</td>
<td>65</td>
<td>41 (63.1)</td>
</tr>
</tbody>
</table>
Detection of the East Asian cagA genotype

The East Asian cagA genotype was detected by performing nested PCR using the DNA samples extracted from the participants’ stool specimens (Fig. 1b, c). First, using two pairs of gene-specific primers, we amplified a region at the 3’-end of the cagA gene that codes for multiple EPIYA phosphorylation motifs. Next, we confirmed the presence of the East Asian cagA genotype by performing two separate rounds of PCR using specific primer pairs (F2 + EA-R and FA-F + R3). As shown in Fig. 1(c), the specificity of the detection method was confirmed by using H. pylori strains with the East Asian cagA genotype as a reference. The East Asian cagA genotype was detected in 41 of 65 (63.1%) genomic DNA samples of H. pylori (Table 3). The incidence of cagA-positive H. pylori ranged from 40.0 to 100.0% across all age groups (Table 2). There was no significant difference between the age groups, except for the group comprising individuals aged ≥60 years, because of the small sample number of participants in this age group.

The results of the present study indicated that 22.0% of the healthy asymptomatic Japanese individuals participating in the study may be infected with the highly virulent H. pylori strain. A considerably higher number of healthy individuals were found to have infection with the highly virulent East Asian cagA-positive H. pylori in Japan than in Thailand [where 2.8% (5/179) healthy asymptomatic individuals were positive for the highly virulent H. pylori infection; unpublished data]. The cause of the highly virulent H. pylori infection in a considerably high number of asymptomatic Japanese individuals is unknown. However, in a recent report, it has been suggested that (1) the geographical distribution of H. pylori strains harboring a certain virulence factor genotype and (2) the incidence of cancer are responsible for the high incidence of H. pylori infection among asymptomatic Japanese individuals (Yamaoka et al., 2008). The findings of Yamaoka et al. (2008) were based on genotype analysis of H. pylori strains that were clinically isolated from patients; however, their finding of a high incidence of gastric cancer in countries where the East Asian CagA is predominant is in agreement with the result obtained in our study.

A recent study showed that the eradication of H. pylori significantly suppressed the development of metachronous gastric cancer (Fukase et al., 2008). The report does not directly suggest that the eradication of H. pylori infection in healthy asymptomatic individuals will suppress the onset of gastric cancer in the future, but it highlights the significance of H. pylori infection in gastric cancer development. Therefore, a silent infection with a highly virulent strain of H. pylori, such as one with the East Asian cagA genotype, in healthy individuals may be a critical public health issue in the prevention of gastric cancer.

To our knowledge, this is the first report on the prevalence of H. pylori infection among healthy asymptomatic Japanese individuals that describes results that were directly revealed by genetic analyses. We found a relatively high incidence of infection with the highly virulent H. pylori strain among asymptomatic adult Japanese individuals. Further investigations with a larger number of participants are required to determine precisely the significance of H. pylori with a virulence factor genotype in the development of gastric cancer.

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REFERENCES


