Evaluation of a stool antigen test using a mAb for native catalase for diagnosis of *Helicobacter pylori* infection in children and adults

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Non-invasive diagnosis of *Helicobacter pylori* infection is important not only for screening of infection but also for epidemiological studies. Stool antigen tests are non-invasive and are convenient to identify *H. pylori* infection, particularly in children. We evaluated the stool antigen test, which uses a mAb for native catalase of *H. pylori* developed in Japan. A total of 151 stool samples were collected from participants (52 children and 99 adults) of the Sasayama Cohort Study and stored between −30 and −80 °C. The stool antigen test used was Testmate pylori antigen (TPAg), and was performed according to the manufacturer’s instructions. Furthermore, we conducted a quantitative real-time PCR test and compared the PCR results with those of the TPAg test. When compared with the results in real-time PCR, the sensitivity of TPAg was 89.5 % overall, 82.7 % for children and 92.4 % for adults, and the specificity was 100 %. The accuracy was 93.4 % overall, 90.4 % for children and 94.9 % for adults, and there was no significant difference in the accuracy of TPAg between children and adults. Five of 28 children (18 %) and five of 38 adults (13 %) were PCR positive with negative TPAg results. Four of five children with positive PCR and negative TPAg results were given a 13 C-urea breath test and all four children tested negative. No significant correlation was observed between the TPAg results and DNA numbers of *H. pylori* in faeces among children or adults. A stool antigen test (TPAg) using a mAb for native catalase is useful for diagnosis of *H. pylori* in children and adults. Additionally, this test has particularly high specificity.

INTRODUCTION

Since the discovery of *Helicobacter pylori* (Marshall & Warren, 1984), many studies have revealed that this bacterium causes gastritis, peptic ulcer diseases, gastric cancer and extragastric diseases such as idiopathic thrombocytopenic purpura or iron-deficiency anaemia (Choe et al., 1999; Emilia et al., 2001; Uemura et al., 2001). *H. pylori* has spread throughout the world as a pathogen, and it was estimated in 2002 that about 60 million Japanese individuals are infected with this pathogen (Asaka, 2002). Recently, the infection rate in children and young adults has been decreasing in Japan. It is not practical to perform an upper gastrointestinal endoscopy for diagnosis of *H. pylori* infection in asymptomatic children or for epidemiological analysis. In addition, serum antibody tests are not recommended for younger children because of their low sensitivity (Leal et al., 2008). A 13C-urea breath test (UBT) can be performed on older children but not on young children (Leal et al., 2011b). The stool antigen test is non-invasive and convenient to assess *H. pylori* infection, particularly for children. The Testmate pylori antigen (TPAg) enzyme immunoassay (EIA) utilizes a mAb to check for native *H. pylori* catalase (Wakamoto Pharmaceutical) with high specificity (Suzuki et al., 2002a, b). In addition, an EIA is used for diagnosis of *H. pylori* and is more useful to determine the results of eradication therapy than a UBT (Shimoyama et al., 2010).

The aim of this study was to examine in children whether a mAb-based stool antigen test is applicable to determine the presence of *H. pylori*. Furthermore we performed quantitative real-time PCR with 16S rRNA gene primers of *H. pylori* using faecal samples from children and adults.
METHODS

Stool samples. We conducted a retrospective study using stored samples from participants of the Sasayama Cohort Study in two consecutive years: 2010 and 2011. In brief, subjects of the study were recruited from seven elementary schools, six kindergartens and three nursery schools in Sasayama city, Hyogo, Japan, over the course of the two years. In 2010, 1299 children aged 0–9 years were asked to give stool samples and 689 participated in the study. In 2011, 835 of the 1909 children asked, aged 0–12 years, participated. Furthermore, we collected 109 stool samples from the staff of the elementary schools, kindergartens and nursery schools, and 103 stool samples were collected from the family members (adults) of the selected children during the course of the study. In this study, faecal specimens excluding diarrhoeal stool samples were collected. None of the adults recruited in this study had previously undergone eradication therapy for H. pylori. A faecal sample (~2–10 g) was collected from each child or adult and stored at between −30 and −80 °C until used. In this study, we chose the TPAg-positive and -negative stool samples from both children and adults.

Informed consent was obtained from all participants or parents. The research protocol was reviewed and approved by the Ethics Committee of the institution.

Stool antigen test. The TPAg EIA (Wakamoto Co.) was used for detection of stool antigen according to the manufacturer’s instructions. Briefly, 30 mg faecal specimen was diluted with 1 ml diluent. Faecal solution (50 µl) of was added to each well and mixed with the reagent. After 30 min incubation at 25 °C, each well was washed five times with washing buffer. Substrate solution (100 µl of 3,3′,5,5′-tetramethylbenzidine with H2O2) was added to each well and mixed for 1 min. The reaction was terminated with 50 µl 0.5 M H2SO4. Absorbance at 450 nm/630 nm was measured by a spectrophotometer and the cut-off value of the test was taken as 0.10.

Bacterial strain and culture conditions. H. pylori TK1402 strain was used as standard strain for quantitative real-time PCR (Osaki et al., 2006). H. pylori TK1402 was cultured under microaerobic conditions (AnaeroPack MicroAero A-28; Mitsubishi Gas Chemical Co.,) at 37 °C on a Brucella plate containing 1.5 % agar and 7 % horse serum for 48 h and inoculated in Brucella broth containing 7 % horse serum. After an 18 h incubation under microaerobic conditions, 1 ml culture (OD600 = 1.0) was collected.

DNA extraction and quantitative real-time PCR. H. pylori DNA was extracted from a cultured strain using a Wizard Genomic DNA Purification kit (Promega). One millilitre of an 18 h bacterial culture containing 1 × 108 c.f.u. H. pylori, quantified by microaerophilic cultivation, was centrifuged. The pellet was resuspended in 480 µl 50 mM EDTA, and 120 µl 400 µg lysozyme ml−1 was added followed by incubation of the mixture at 37 °C for 60 min. Subsequent steps were performed according to the manufacturer’s instructions. Finally, 100 µl nuclease-free water was used for elution of the purified DNA.

DNA from stool samples was isolated using a QIAamp Stool kit (Qiagen) according to manufacturer’s instructions, with some modifications. Briefly, 180–200 mg frozen faeces was suspended into 450 µl buffer ASL with 0.3 g glass beads (GB-01, diameter 0.1 mm; TOMY), and the suspension was mixed vigorously for 30 s using a Multi-beads Shocker (MB755U; Yasui Kikai) at 1500 r.p.m. three times at 30 s intervals. After a 5 min incubation at 75 °C, the suspension was mixed again in the same manner. After centrifugation at 14 000 g for 5 min, subsequent steps were performed according to the manufacturer’s instructions. Two hundred microlitres of the DNA solution in buffer AE was eluted at the final step.

Quantitative real-time PCR of confirmed H. pylori antigen-positive faecal samples was done using H. pylori 16S RNA gene-specific primers (16S2-F: 5′-CGCTAAAGATCAGCTGTATGTCC-3′; 16S2-R: 5′-CCGTGTCAGGTCAGTGTGT-3′) (Osaki et al., 2012). The PCR was performed in duplicate or triplicate. First, two reaction wells were used for each sample. If the two wells showed positive or negative reactions, the PCR result was determined as positive or negative, respectively. If the two wells showed opposite results (positive and negative), the PCR was performed again using one more well. In this case, a positive result was determined according to at least two positive reactions for the sample in the PCR. Quantitative data were calculated from a standard curve generated by amplifying serial dilutions of a known quantity of ampiclon. To confirm the specificity of the PCR product, a melting-curve analysis was done after amplification to distinguish the target PCR product from the non-target PCR product. The melting curves of PCR products were obtained by slow heating at temperatures in dissociation steps. The melting temperature (Tm) value of control strains was used to confirm PCR specificity. It was shown that the data with standard Tm ± 1 °C were the same as the control product.

UBT. Children were fasted at least 4 h before the UBT and breath samples were collected before and 20 min after ingestion of 13C-urea. The dosage of 13C-urea was 100 mg for all ages. An infrared spectrometer (UBiT- IR300; Otsuka Electronics Co.) was used in this study and an increase of more than 3.5 % was considered positive (Kato et al., 2002).

Statistical analysis. Analyses were carried out separately among children and adults, and combined when necessary. Agreement between positive or negative results of the TPAg and PCR was evaluated. TPAg results were classified into three levels, and the mean DNA number of H. pylori in faeces was calculated for each level. Correlation between results of the TPAg and PCR was evaluated by calculating Spearman’s rank correlation coefficient.

RESULTS

In the 2010 group, 13 and 676 children were identified as TPAg positive and negative, respectively. In the 2011 group, 15 and 820 children were identified as TPAg positive and negative, respectively. We chose 24 random samples (from children aged 2–12 years, mean 5.9 ± 2.0) of 28 positives and 28 random samples (from children aged 2–8 years, mean 5.0 ± 1.7) of 1496 negatives, and performed quantitative real-time PCR using 16S rRNA gene primers of H. pylori.

Of the stool samples obtained from the staff, 29 of 109 samples were positive by the TPAg. Concerning family members, 42 were positive and 61 were negative in the TPAg. Sixty-one of 71 positive stool samples and 38 of 141 negative stool samples obtained from adults (staff and family members) were chosen and used for quantitative real-time PCR using 16S rRNA gene primers of H. pylori.

The correlation between the results from the TPAg and PCR tests is presented in Table 1. All TPAg-positive samples from both children and adults were also shown to be positive by PCR. Five of 28 children (18 %) and five of 38 adults (13 %) revealed PCR-positive results with negative TPAg results. When compared with the results in real-time PCR, the sensitivity of TPAg was 89.5 %
the sensitivity and specificity of the TPAg were 89.5% using PCR as a standard, it was demonstrated that and the results of the UBT agreed with those of the TPAg. Overall, 82.7% for children and 92.4% for adults, and the specificity was 100%. The accuracy was 93.4% overall, 90.4% for children and 94.9% for adults, and there was no significant difference in the accuracy of the TPAg between children and adults. Among the subjects, four children with positive PCR and TPAg results were given a UBT and were found to be positive (Table 2). Four of five children with positive PCR and negative TPAg results were given a UBT and all four were negative (Table 2).

The relationship between TPAg values and the numbers of the H. pylori quantified by quantitative real-time PCR results and the DNA numbers of H. pylori in faeces is presented in Fig. 1. When samples were limited to the PCR-positive results, no significant correlation was observed between TPAg values and the numbers of H. pylori in faeces among children or adults.

**DISCUSSION**

In the present study, the accuracy of the TPAg for diagnosis of H. pylori infection in children was investigated and compared with the PCR results using H. pylori 16S rRNA gene-specific primers. All TPAg-positive stool samples agreed with the results of the PCR. However, in some cases, there were discrepancies in the test results between the TPAg and PCR results. In these cases, a UBT was performed for some of the children with discrepant results, and the results of the UBT agreed with those of the TPAg. Using PCR as a standard, it was demonstrated that the sensitivity and specificity of the TPAg were 89.5% (85/95) and 100% (56/56), respectively. However among PCR-positive children or adults, a significant correlation was not observed between the TPAg values and the numbers of H. pylori in faeces.

The stool antigen test is non-invasive and convenient because of the ease of obtaining samples. It is used not only for diagnosis but also for epidemiological studies of H. pylori in children. Several types of stool antigen test have been used for the diagnosis of H. pylori infection, and in these tests EIA or immunochromatography is used with polyclonal or mAbs. However, the guidelines of the European Helicobacter Study Group state that the diagnostic accuracy of the stool antigen test is equivalent to the UBT if a validated laboratory-based mAb test is used (Malfertheiner et al., 2012). In addition, the Japanese guidelines for the management of H. pylori infection also recommend the use of mAb stool antigen tests for confirming infection (Asaka Leal, 2010).

Native catalase was identified as an antigen produced by H. pylori (Suzuki et al., 2002a, b) and the TPAg uses a mAb against catalase. The TPAg does not react with bacterial antigens of other Helicobacter species or intestinal bacteria, whereas it reacts with antigens from most H. pylori clinical isolates (Sato et al., 2012). The TPAg has been prepared for two types of testing: rapid TPAg (immunochromatography) and TPAg EIA. The rapid TPAg is reported to have a high accuracy for the diagnosis of H. pylori infection in asymptomatic children (Cardenas et al., 2008), but a meta-analysis to evaluate the performance of stool antigen tests showed a low accuracy of one-step mAb tests (Leal et al., 2011a). There are no reports concerning the TPAg EIA using a mAb to catalase for children. We used the stool antigen test for epidemiological study of H. pylori in Japanese children (Okuda et al., 2001). In Japan, the prevalence of H. pylori infection has become particularly low (~5%) (Okuda et al., 2001) and therefore a good diagnostic method to reduce incidences of false positives is needed. It is well known that quantitative real-time PCR is a very sensitive test to detect low numbers of H. pylori, regardless of viability. According to the results by the UBT for four children with TPAg-negative and PCR-positive results, it was clarified that the PCR-positive results were false positives. In contrast, the TPAg is not as sensitive as PCR in terms of detection of small numbers of H. pylori. The exact reason why 10 specimens from five children and five adults with TPAg-negative results were positive in the PCR test is not known, but it is possible that the PCR detected very low numbers of H. pylori or dead H. pylori, or transit passage of H. pylori without persistent infection in gastric mucosa.

In this study, we evaluated a TPAg comparing PCR using H. pylori 16S rRNA gene-specific primers. All 85 TPAg-positive stool samples obtained from 24 children and 61 adults agreed with the results of the PCR with 100% specificity. This showed that the TPAg is an excellent method for epidemiological studies in low-prevalence area. However, 10 (five children and five adults) of the 66 TPAg-negative stool

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**Table 1. Correlation of the results of TPAg and PCR tests**

<table>
<thead>
<tr>
<th></th>
<th>Children</th>
<th></th>
<th>Adults</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PCR (+)</td>
<td>PCR (−)</td>
<td>PCR (+)</td>
</tr>
<tr>
<td>TPAg (+)</td>
<td>24</td>
<td>0</td>
<td>61</td>
</tr>
<tr>
<td>TPAg (−)</td>
<td>5</td>
<td>23</td>
<td>5</td>
</tr>
<tr>
<td>Total</td>
<td>29</td>
<td>23</td>
<td>66</td>
</tr>
</tbody>
</table>

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**Table 2. Comparison of the results of TPAg, PCR and UBT tests for eight children**

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Age (years)</th>
<th>TPAg (OD)</th>
<th>H. pylori DNA (g⁻¹)</th>
<th>UBT (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>519</td>
<td>5</td>
<td>0.438</td>
<td>7037</td>
<td>32.7</td>
</tr>
<tr>
<td>509</td>
<td>5</td>
<td>0.790</td>
<td>3696</td>
<td>27.3</td>
</tr>
<tr>
<td>714</td>
<td>7</td>
<td>1.153</td>
<td>92686</td>
<td>25.4</td>
</tr>
<tr>
<td>X01</td>
<td>12</td>
<td>0.402</td>
<td>19023</td>
<td>33.9</td>
</tr>
<tr>
<td>325</td>
<td>3</td>
<td>0.019</td>
<td>7149</td>
<td>1.1</td>
</tr>
<tr>
<td>504</td>
<td>5</td>
<td>0.016</td>
<td>19811</td>
<td>1.5</td>
</tr>
<tr>
<td>631</td>
<td>6</td>
<td>0.021</td>
<td>5552</td>
<td>1.9</td>
</tr>
<tr>
<td>651</td>
<td>6</td>
<td>0.013</td>
<td>5574</td>
<td>0.0</td>
</tr>
</tbody>
</table>
samples were positive by PCR. This may show a low sensitivity of the TPAg, but we conducted a UBT for four of the six children with discrepant results from the TPAg-negative stool samples. The results of the UBT agreed with the results of the TPAg. As PCR can detect very low quantities of H. pylori DNA, this discrepancy may be due to transit passage of H. pylori in the intestinal tract. Ou et al. (2013) also reported that gastric mucosa fluorescence quantitative PCR (fqPCR) is more sensitive than routine histology, a rapid urease test, UBT alone or UBT in combination to detect H. pylori infection in children. One hundred and thirty-eight patients who underwent gastroscopy and a UBT were included in the study. Using the gastric mucosa fqPCR method of testing, 38 ‘H. pylori positive patients’ tested positive, and additionally, eight (8%) of the 100 ‘gold standard negative’ children also tested positive (Ou et al., 2013).

We have previously reported that intra-familiar infection of H. pylori is possible by mother-to-child or father-to-child transmission in three families by multilocus sequencing type analysis using faecal specimens (Osaki et al., 2013). Furthermore, by using DNA specimens extracted from the H. pylori isolates, it was shown that the mother or father might be the main origin of intra-familiar infection of H. pylori (data not shown).

In this study, the correlation between TPAg values and the number of H. pylori evaluated by quantitative real-time PCR was examined, but no correlation was observed. It is likely that this result was based on the higher sensitivity of the quantitative real-time PCR assay for detection of H. pylori. It is also possible that the non-correlation between the results in the TPAg and real-time PCR might be due to the difference in the target molecules in these detection methods. In four representative cases with TPAg-positive/PCR-positive and four cases with TPAg-negative/PCR-positive results, the TPAg matched the UBT results. The TPAg is a clinically useful test in terms of its ease to perform.

Conclusion

In the present study, a stool antigen test (TPAg) using a mAb for native catalase was evaluated for diagnosis of H. pylori in children and adults, and the results were compared with those from a quantitative real-time PCR assay. It was demonstrated that the mAb-based TPAg had high sensitivity and 100% specificity, indicating that TPAg is a useful method to detect H. pylori not only in adults but also in children.

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