Serological Differentiation of *Helicobacter pylori* CagA(+) and CagA(−) Infections

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Abstract. Many *Helicobacter pylori* strains causing gastroduodenal diseases have a *cagA* gene encoding CagA protein, a virulence factor of these bacteria. Anti-CagA antibodies produced by the majority of people infected with CagA(+) strains can indicate such an infection. In this study, the efficacy of three immunoenzymatic tests for detecting CagA(+) and CagA(−) infections were compared: immunoblot (Milenia™ID Blot *H. pylori* IgG; MB) and ELISA conducted either with a recombinant immunodominant fragment of CagA (rCagA) or the full-length CagA molecule (flCagA). The ¹³C-urea breath test (¹³C-UBT) was used for establishing *H. pylori* status. The serum samples from 157 individuals were used for serodiagnosis. *H. pylori* CagA(+) infection was detected in *H. pylori*-infected individuals with similar frequencies by MB (64%) and flCagA-ELISA (60%) and a little less frequently by rCagA-ELISA (53%). There was a high coincidence between the negative results of these three tests for *H. pylori*-uninfected individuals with no anti-CagA IgG in the serum (96–100%). The results show that rCagA-ELISA and, especially, flCagA-ELISA are easy, inexpensive and useful noninvasive assays for the discrimination of CagA(+) and CagA(−) *H. pylori* infections in subjects examined by urea breath test.

Key words: *Helicobacter pylori*; CagA; serodiagnosis.

Introduction

*Helicobacter pylori* is a major cause of gastroduodenal diseases in humans, including chronic gastritis, ulcers and gastric malignancies¹¹,¹³. However, the majority of *H. pylori* infections are asymptomatic.

Vacuolating cytotoxin (VacA), a secreted protein with a mass of about 90–95 kDa, and the CagA antigen, the cytotoxin-associated gene A protein of 128–145 kDa, are the most important *H. pylori* virulence agents ⁵, ⁹, ²¹. The *cagA* gene is a marker for a 40 kb pathogenicity island, PAI, present only in the type I *H. pylori* strains VacA(+)/CagA(+), but not in the type II strains VacA(+)/CagA(−). ⁴, ²⁵. Several PAI genes are involved in the secretion of interleukin 8 (IL-8) by human epithelial cells infected with CagA(+) *H. pylori* strains¹. IL-8 is implicated in the inflammatory response of gastric mucosa to *H. pylori* infection¹¹. CagA is translo-
cated into human gastric cells by the type IV secretion apparatus of *H. pylori*. This may have important biological consequences in chronic infections. Type 1 *H. pylori* strains have been isolated more frequently from patients with duodenal ulcers and gastric tumors. For this reason, noninvasive test allowing the discrimination of infections from CagA(+) and CagA(−) *H. pylori* bacteria are needed. *H. pylori* infections themselves may be detected by culture, urease and histological examination of gastric biopsies. Also, polymerase chain reaction (PCR) for the genes encoding bacterial urease and CagA in gastric tissues may be useful. However, all these procedures are invasive and cannot be recommended for the examination of the general population and should be avoided in children. In comparison, the 13C-urea breath test (13C-UBT) is noninvasive and adequate for the identification of *H. pylori* infected subjects; however, it is not suitable for the discrimination of CagA(+) and CagA(−) *H. pylori* infections.

Almost all *H. pylori*-infected patients produce antibodies against these bacteria. Detection of such antibodies has been used in routine diagnosis. The CagA surface antigen of type 1 *H. pylori* strains is highly immunogenic. Therefore, serum anti-CagA IgG may indicate a chronic infection from CagA(+) *H. pylori* bacteria.

The aim of this study was to compare the utility of an in-house, indirect immunoenzymatic test, ELISA, conducted with two CagA preparations (a recombinant immunodominant fragment of CagA – rCagA, and full-length CagA molecule – flCagA) and a commercial Western blot kit (Milenia®ID Blot *H. pylori* IgG, DPC Biermann GmbH, Bad Nauheim, Germany; MB) for detecting serum IgG to *H. pylori* antigens including the 120 kDa protein (CagA).

Materials and Methods

Subjects. One hundred and fifty-seven individuals (97 subjects with dyspeptic symptoms and 60 asymptomatic volunteers), consisting of 68 males and 89 females aged 5 to 69 (mean age 23.3 ± 14.8) were included in this study, which was approved of by the local Ethics Committee. All participants gave written informed consent. In all subjects, capsule-based 13C-UBT was performed as the “noninvasive standard” for detecting *H. pylori* infection on the basis of urease activity. Blood was obtained from all individuals for serological examination.

Capsule-based 13C-UBT. The urea-breath test was performed as previously described using 13C-urea (MassTrace Inc., MA, USA). Patients and volunteers were asked to come for examination after overnight fasting. The exclusion criteria for the test were: pregnancy, previous gastric surgery and the recent use of medications such as bismuth and antibiotics (within the past month), or sucralfate, proton pump inhibitors and histamin receptor antagonists (within the past 2 weeks). The breath sample was collected and analyzed by a 13C analyser (Europa Scientific, UK). As previously mentioned 13C-UBT results correlated highly with the combined results of the rapid urease test (RUT) and histology for *Helicobacter*-like organisms (HLO) in gastric biopsies. The 13C-UBT was 97% sensitive, 95% specific, with a positive predictive value of 90% and a negative predictive value of 98% for the detection of *H. pylori* infection in humans.

Milenia®ID Blot *H. pylori* IgG. MB (DPC Biermann GmbH, Bad Nauheim, Germany) was performed according to the producer’s protocol. The reactivities of human IgG with highly specific (CagA – 120 kDa, VacA – 87 kDa, urease subunits UreA – 29 kDa, UreB – 66 kDa), specific (35, 26, 20 kDa proteins) and non-specific (heat shock protein (Hsp) 60 kDa, flagellin (Fla) 54, 14 kDa protein) *H. pylori* antigens were determined. The results were interpreted as positive (confirming *H. pylori* infection) when at least two highly specific bands were stained; equivocal when at least one highly specific and two specific bands were stained, and negative (*H. pylori* infection excluded) when no highly specific or specific bands were stained (only some staining with non-specific antigens was observed). The sensitivity and specificity of the MB test in correlation with 13C-UBT were 94 and 86%, respectively, with positive and negative predictive values as high as 85 and 95%.

ELISA. Serum anti-*H. pylori* CagA IgG were determined by ELISA as previously described, with several modifications. Microplates (Nunc-Immuno Plate MaxiSorp, Nunc, Kastrup, Denmark) coated with 1 μg/ml of rCagA or 1 μg/ml of flCagA protein (from the Institute of Immunobiology in Siena, Italy) in 0.05 M carbonate buffer, pH 9.6, were used. The sera were diluted 1:500 and peroxidase conjugated rabbit anti-human IgG antibodies (Dako, Glostrup, Denmark) 1:6000. The o-phenylenediamine dihydrochloride (Sigma, St. Louis, USA) was used as a chromogen. The optical density (OD) values were read at 450 nm (1420 Victor 2, Oy, Turku, Finland). The assay was calibrated with standard sera from subjects infected with *H. pylori* CagA(+) (2 sera) or *H. pylori* CagA(−) (2 sera) diluted 1:500. *H. pylori* CagA(+) or CagA(−) infections were
confirmed by cagA PCR in gastric biopsy\textsuperscript{18}. A panel of negative control samples formed the basis for determining the cutoff points between positive and negative tests. A positive cutoff was established as two standard deviations (SD) above the mean of two control negative sera from subjects uninfected with \textit{H. pylori}\textsuperscript{3}. For rCagA-ELISA the OD cutoff value was 0.13 and for fICagA-ELISA 0.14.

The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy of rCagA-ELISA and fICagA-ELISA assays were defined in correlation with the result of MB detecting IgG to CagA. Receiver operator characteristic curve analysis was also performed\textsuperscript{6}.

Results

Determination of \textit{H. pylori} status

The capsule-based \textsuperscript{13}C-UBT, detecting \textit{H. pylori} urease activity, was used as the “noninvasive standard” for establishing \textit{H. pylori} status.

A total number of 157 individuals was included in this study. Seventy individuals (45\%) were positive for \textit{H. pylori} by \textsuperscript{13}C-UBT (69 out of the 70 individuals suffered from dyspepsia). For 87 of the 157 subjects (55\%), results of the test were negative (43 of the 87 \textsuperscript{13}C-UBT(–) individuals had dyspeptic symptoms). The cases with dyspepsia were referred to endoscopy. There was complete coincidence between positive or negative results of \textsuperscript{13}C-UBT and positive or negative results of endoscopy-based invasive methods (RUT and HLO) in dyspeptic patients.

\textit{Discrimination of CagA(+) and CagA(–) \textit{H. pylori} infections by Western blot and ELISA systems}

In this study, discrimination of \textit{H. pylori} CagA(+) and CagA(–) \textit{H. pylori} infections was based on three noninvasive serological tests which detect serum IgG to CagA protein: MB with the CagA band, rCagA-ELISA and fICagA-ELISA.

In the group of 70 \textsuperscript{13}C-UBT(+) subjects, the frequency of CagA(+) \textit{H. pylori} infections detected by MB, rCagA-ELISA and fICagA-ELISA was 64\% (45 sera), 53\% (37 sera) and 60\% (42 sera), respectively (Fig. 1, Table 1). The coincidence between positive results of MB and rCagA-ELISA and fICagA-ELISA was 82 and 93\%, respectively. The coincidence between rCagA- and fICagA-ELISA was 88\%. Anti-CagA IgG

\begin{figure}[h]
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\caption{The frequency of serum IgG to CagA detected by: immunoblot – Milenia\textsuperscript{\textregistered} ID Blot \textit{H. pylori} IgG (MB), rCagA-ELISA and fICagA-ELISA in the individuals examined by urea-breath test (\textsuperscript{13}C-UBT). rCagA – recombinant CagA (immunodominant fragment), fICagA – full-length CagA molecule}
\end{figure}
were not detected by MB, rCagA-ELISA and flCagA-ELISA for 25 (36%), 33 (47%) and 28 (40%) of the 70 H. pylori 13C-UBT(+) subjects, respectively (Fig. 1, Table 1).

Complete coincidence (100%) between negative results of MB and flCagA-ELISA was found for 75 (86%) of the 87 individuals with H. pylori infection excluded by 13C-UBT (Fig. 1, Table 1). These and three additional individuals were negative for anti-CagA IgG by rCagA-ELISA. In 13C-UBT(−) subjects the coincidence between negative results of rCagA-ELISA and MB or rCagA-ELISA and flCagA-ELISA (excluding H. pylori CagA(+) infection) was 96%. IgG to CagA were detected by MB and flCagA-ELISA for 12 (16%) of the 75 individuals with negative 13C-UBT result and for 9 (12%) subjects by rCagA-ELISA (Fig. 1, Table 1).

For the epidemiological validation of the ELISA assays, 120 serum samples were selected: sera from H. pylori-infected individuals producing IgG to CagA detected by MB (45 sera), and sera from H. pylori-uninfected subjects with no anti-CagA IgG detected by MB (75 sera). The rCagA-ELISA was 84% sensitive and 96% specific. The PPV and NPV were 93 and 91%, respectively, and the accuracy was 92% (for an OD cutoff of 0.13). With an OD cutoff of 0.14, flCagA-ELISA was 93% sensitive and 89% specific with an accuracy of 91%, and with PPV and NPV at 84 and 96%, respectively.

Discussion

Invasive methods based on endoscopy, which are accepted as the "gold standard", are not suitable for mass screening and are not recommended to children. Due to the patchy character of H. pylori colonization, detection of bacteria can fail with invasive methods. Difficulty in culturing H. pylori reduces the sensitivity of endoscopy-based tests. A well-accepted diagnostic procedure is the urea breath test (highly sensitive and specific), which detects the activity of bacterial urease produced by all H. pylori strains. However, this test is not suitable for the discrimination of CagA(+) and CagA(−) H. pylori strains. This is an important diagnostic task because CagA(+) type I strains induce more severe diseases, i.e. duodenal ulcer and gastric cancer11,22. Therefore, subjects infected with CagA positive strains should be monitored more rigorously.

Using immunoblot (MB), we determined the interaction of anti-H. pylori IgG present in dyspeptic patients and healthy individuals with highly specific (CagA – 120 kDa, VacA – 87 kDa, subunits UreA – 29 kDa, UreB – 66 kDa), specific (proteins of 26, 20 and 35 kDa) and non-specific (Hsp 60 kDa, Fla 54, 14 kDa protein) antigens of these bacteria. Among the 70 13C-UBT(+) subjects, 66 had anti-H. pylori IgG detected by MB. IgG to CagA were detected by MB, rCagA-ELISA and flCagA-ELISA for 12 (16%) of the 75 individuals with negative 13C-UBT result and for 9 (12%) subjects by rCagA-ELISA (Fig. 1, Table 1).

For not one of the 21 13C-UBT(+) subjects with IgG to H. pylori highly specific and specific antigens, IgG to CagA were detected by MB, rCagA-ELISA and flCagA-ELISA for 45, 37 and 42 out of 66 individuals with anti-H. pylori IgG (Table 1). For not one of the 21 13C-UBT(+) subjects with IgG to H. pylori highly specific and specific antigens, IgG to CagA were detected by MB, rCagA-ELISA and flCagA-ELISA. One out of 4 subjects negative for anti-H. pylori IgG and anti-CagA IgG in MB was positive for IgG to CagA by rCagA-ELISA and flCagA-ELISA. A possible explanation for the lack of specific anti-H. pylori IgG in these 4 patients could be the stage of infection. The development of humoral response during an infection with regard to antibody isotype, titer and avidity depends on the antigenic structure of the microbe, the density and kinetics of colonization, and the age of the infected host. The first step of acute H. pylori infection is correlated with the production of specific IgM class antibodies. That is why the detection of H. pylori infection on the basis of anti-H. pylori IgG can be sometimes missed. The detection of IgG to CagA in one of the 4 subjects with no anti-H. pylori IgG and no anti-CagA IgG in MB could be a test error. It might also be explained by differences in the CagA molecules used in the MB and ELISA assays. However, other unknown reason for this discrepant result cannot be excluded.

Table 1. Discrimination of H. pylori CagA(+) and CagA(−) infections by ELISA and Milenia® ID Blot H. pylori IgG (MB) methods

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<tr>
<th>Group investigated</th>
<th>MB</th>
<th>ELISA MB</th>
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<td>anti-H. pylori IgG</td>
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<td>anti-H. pylori IgG</td>
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<td>1. CagA(−) infection</td>
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<td>2. CagA(−) infection</td>
<td>93% sensitive</td>
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In the group with negative 13C-UBT results, excluding H. pylori infection, the IgG to CagA were detected for 12 of the 87 individuals by both MB and fICagA-ELISA. Nine of these 12 subjects were also positive for anti-CagA IgG by rCagA-ELISA. All these subjects produced IgG reacting with highly specific and specific H. pylori antigens, detected by MB. It is possible that H. pylori infection was missed in these cases by 13C-UBT. This might be due to ingestion by the patients, especially adults, of some medications which could affect the 13C-UBT result. Some discrepancies in the tests for detection of H. pylori could be due to infections caused by new Helicobacter species, such as H. hepaticus or H. bilis, or unrelated microbes. It has been shown that the antigens of those two Helicobacter species may crossreact with H. pylori antigens in ELISA. However, up to now cross-reactivity with H. pylori infections caused by CagA(+) strains, especially in light of the reactivity of their sera with highly specific and specific H. pylori antigens.

In conclusion, this study shows that ELISA for the detection of serum IgG to CagA is a rapid, noninvasive, inexpensive test which appears to be efficacious in the differentiation of H. pylori CagA(+) and CagA(-) infections in subjects examined by the urea breath test. Compared with other reports, the rCagA-ELISA and fICagA-ELISA were shown to have sufficient diagnostic ability for the discrimination of CagA(+) infections (84 and 93% sensitivity, 96 and 89% specificity, 92 and 91% accuracy, respectively) and of CagA(+) strains, especially in light of the reactivity of their sera with highly specific and specific H. pylori antigens.

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References


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