Combined serum IgG response to Helicobacter pylori VacA and CagA predicts gastric cancer

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VacA; Western blot; immunoblot.

Abstract
Helicobacter pylori is a major factor for the development of gastric cancer. The aim of this study was to define serum antibody patterns associated with H. pylori infection in patients with gastric cancer using a Western blot technique. Serum samples collected from 115 patients with gastric cancer and 110 age- and gender-matched patients without gastrointestinal diseases were tested for IgG antibodies to H. pylori antigens (outer membrane proteins and whole cell preparations). No significant differences were found between patients with and without gastric cancer using outer membrane proteins (82% and 73%, P > 0.05) or whole cell antigens (84% and 76%, P > 0.05), respectively. The significant differences between patients with and without gastric cancer were associated with bands of 94 kDa (54% and 20%, P < 0.001) and 30 kDa (65% and 44%, P < 0.01). A combination of antibodies to 85 kDa (VacA) and 120 kDa (CagA) was significantly (P < 0.01) more frequent in gastric cancer patients than in patients without gastric cancer. The detection of antibodies to 94- and 30-kDa bands, in association with the determination of serum antibodies to CagA/VacA, may have a prospective value in assessment of the risk of developing of gastric cancer.

Introduction
Gastric cancer is the fourth most common cancer and the second most common cause of cancer deaths worldwide (Parkin et al., 2001). Although a multifactorial etiology of gastric cancer is acknowledged, the main factors are infection with H. pylori and the inadequate intake of antioxidant micronutrients (Correa, 2005). Helicobacter pylori is a spiral-shaped bacterium that colonizes the human gastric mucosa and infects more than 50% of the world’s population. This microorganism is associated with an increased risk of gastrointestinal diseases (Feldman, 2001) and is classified as a group I carcinogen (IARC, 1994). Cancer risk is believed to be related to differences in H. pylori strains. It is particularly enhanced for individuals infected with H. pylori carrying the cag island, a 40-kb locus that identifies a strain of H. pylori that has an intimate interaction with stomach epithelium (Tomb et al., 1997). The cagA gene has been detected in about 60–70% of H. pylori strains (Covacci et al., 1993), but in high gastric cancer risk populations the proportion is much higher, c. 90%. The vacA gene is present in all H. pylori strains, but only 50–65% of strains produce the cytotoxin and induce vacuolization of HeLa cells in vitro and gastric epithelial cells in vivo (Cover et al., 1993). Serological studies have shown that H. pylori strains expressing the CagA or VacA proteins have been associated with a stronger inflammatory reaction and have been frequently detected in patients with gastric cancer and other gastrointestinal diseases (Held et al., 2004; Sokic-Milutinovic et al., 2004; Hatakeyama & Higashi, 2005; Palestro et al., 2005). The aim of the present study was to examine the prevalence of antibodies to CagA, VacA and other H. pylori antigens in patients with gastric carcinoma and in age- and gender-matched patients without gastrointestinal diseases using the Western blot technique to explore the role of these antigens as risk factors for gastric cancer in Lithuania.

Materials and methods
The study was approved by the local medical ethics committee of the University Hospital of Kaunas, Lithuania.

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Patients

Serum samples were obtained from 115 Lithuanian patients with histologically proven noncardiac gastric cancer (66 men and 49 women; mean age 56.9 years; range 30–89 years) who were referred to the Oncology Centre of Lithuania. Tumour staging was based on a histopathological (pTNM) classification system (Hermanek et al., 1997). History of peptic ulcer disease, gastritis, gastrointestinal surgery, and active symptoms of abdominal pain or other gastrointestinal diseases or dyspeptic complaints were recorded.

The other investigative group consisted of 110 hospitalized age- and gender-matched noncancer patients (54 men and 56 women; range 30–89 years, mean age 57.2 years), admitted to the Kaunas Medical University Hospital for disorders unrelated to gastrointestinal diseases, namely, diabetes mellitus, osteoporosis, miscellaneous gynaecological disorders, pneumonia, hemiparesis, glaucoma, cataract, coronary heart disease, and served as control. None of these patients was treated for eradication of H. pylori before enrolment.

Serum samples

Venous blood was collected from the gastric cancer and asymptomatic individuals over a period of 8 months. Serum samples were stored at −20 °C until use.

Antigen preparation for Western blotting

Two different antigen preparations, a crude whole cell antigen and a semi-purified outer membrane protein, were prepared.

Whole cell antigen preparations. Selected bacterial strains (CH 20249, CCUG 17874), grown on chocolate agar, were harvested and washed twice in sterile distilled water. The preparations were centrifuged (7000 g for 10 min), bacterial pellets stored at −20 °C and resuspended in sterile water to a concentration of 0.5 g wet weight mL⁻¹ of sterile water. Whole cell preparations (0.5 g wet weight mL⁻¹ of sterile water) were sonicated (20 000 Hz for 45 s), and the procedure repeated five times with a sterile probe (Rapidis 300, 19-mm probe with a 9.5-mm tip). The preparations were cooled during sonication by immersion in ice water. The sonicated bacteria were stored at −20 °C until use.

Outer membrane proteins were prepared as described by Lelwala-Guruge et al. (1992). In brief, H. pylori strain CCUG 17874 was grown on GAB/Camp agar plates for 2 days under microaerophilic conditions at 37 °C. The bacterial culture was harvested, the cell mass washed in phosphate-buffered saline (PBS) (pH 7.2) and resuspended in 0.2 M glycine-HCl buffer (pH 2.2), stirred for 15 min at room temperature, and the cells removed by centrifugation (12 000 g for 15 min at 8 °C). Supernatants were neutralized (pH 7.2) with 1 M NaOH and dialyzed overnight against PBS (pH 7.2) at 4–8 °C. Seven batches were prepared, pooled and preparations stored at −90 °C. This preparation consisted mainly of cell surface proteins (Lelwala-Guruge et al., 1992).

Whole cell–Western blot

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot were carried out as described by Andersen & Espersen (1992). The SDS gel was made of 15% lower separating gel (for two gels; 10 mL distilled water, 10 mL lower Tris buffer, 20 mL bis-acrylamide, 20 μL Temed, 140 μL 10% ammonium persulphate and 60 μL 0.1% riboflavin) and 5% gel (for two gels; 7.1 mL distilled water, 3.0 mL upper TRIS buffer, 2.2L bis-acrylamide, 23 μL Temed, 75 μL 10% ammonium persulphate and 75 μL, 0.1% riboflavin). The whole cell preparation, consisting of 50% sonicated bacteria and 50% whole cells, was mixed 1:1 with sample buffer containing 0.4% sodium dodecyl sulphate and 4.8% d-thioglycollic acid. The suspension was boiled in a water bath for 5 min with low molecular weight standard diluted 1:2000 in sample buffer (Bio-Rad, with protein bands of 14.5, 21.5, 31, 45, 66, and 97.4 kDa). The suspension was poured on the gels and electrophoresis was carried out at 70 V and 500 mA for 20–24 h (1800 V h).

The separated proteins were transferred from the gel to a nitrocellulose membrane at 30 V overnight, in transfer buffer [Tris-hydrochloride (25 mM)-glycine (0.192 M; pH 8.4) containing 4.9 M methanol]. Unspecified binding was blocked by adding 0.5% Tween 20 for 30 min, and the nitrocellulose papers were then cut into 6–7-mm-wide strips and incubated with serum samples diluted 1:500 in Tris-HCl buffer, containing 0.5% Tween 20. All serum samples were left to incubate for 1 h and then washed three times for 10 min in Tris-HCl buffer containing 0.5% Tween 20. Rabbit anti-human IgG antibodies conjugated with horseradish-peroxidase (DAKO, No. 214, Copenhagen, Denmark) were diluted 1:2000 in Tris-HCl buffer containing 0.5% Tween 20 and the nitrocellulose strips incubated in the suspension for 1 h and then washed three times for 10 min as before. A solution containing diocytliumtresulphosuccinate (DONs), tetramethylbenzidine (TMP), citrate-phosphate buffer (pH 5.0) and H₂O₂ was used to stain the nitrocellulose strips and the enzyme reaction was stopped after 3–6 min with a solution of distilled water and DONs. To stain the molecular weight standard, the strips were washed as described above, rinsed with distilled water and then left to incubate in gold staining solution for 1 h.

Antibody reactivity to the high molecular mass bands (VacA 87 kDa or CagA 120 kDa) and low molecular mass bands (15–30 kDa) was analyzed. The immunoblot was
considered *H. pylori*-positive when at least one high- or at least one low molecular mass band was present (Fig. 1).

**Outer membrane protein--Western blot**

Immunoblot analysis for the detection of antibodies was performed as described by Nilsson *et al.* (1997). SDS-PAGE was performed using Protean II Cell Vertical Electrophoresis equipment (Bio-Rad, Richmond, CA). Acid–glycine-extracted proteins from seven pooled antigen batches of *H. pylori* strain NCTC 11637 were separated in a gradient gel (5–20%) and with a 5% stacking gel. The antigen (125 μg gel⁻¹) was diluted in a sample buffer (0.5 M Tris-HCl, pH 6.8, 0.5% Bromophenol blue, 8% glycerol, 4% SDS, 4% 2-mercaptoethanol) and heated at 95 °C for 3 min. After cooling, the proteins were loaded on the gel and separated for 16 h at 80 V. Molecular mass standards (Promega, Scandinavian Diagnostic Services, Falkenberg, Sweden) with proteins ranging from 14.3 to 97.4 kDa were treated similarly.

Separated proteins were transferred electrophoretically to a polyvinylidene difluoride (PVDF) membrane (pore size, 0.45 μm) in a semidyry electrophoresis apparatus (Ancos, Vig, Denmark) for 1 h at a constant current of 0.8 mA cm⁻². The membrane was saturated in blocking buffers I and II. Saturated membranes were rinsed once for 10 min in a washing buffer and cut into strips (blocking and washing buffers were from M. Rucheton, Orstom Laboratories, Montpellier, France). The strips were incubated with sera, diluted 1:100 in washing buffer, and agitated gently for 16 h at 4 °C. Strips were then rinsed three times for 5 min and incubated for 2 h at 4 °C with horseradish peroxidase-labelled anti-human IgG antibodies (DAKO A/S, Glostrup, Denmark) diluted to 1:1600; bound antibodies were detected with a carbazole-acetate buffer (Merck, Darmstadt, Germany).

Antibody reactivity to the high molecular mass bands (120 kDa, 94 kDa, 85 kDa) and low molecular mass bands (30 kDa, 29 kDa, 26 kDa) was analyzed. The immunoblot was considered *H. pylori*-positive when at least one high molecular mass band and/or at least two to five low molecular mass bands were seen (Fig. 2).

**Statistical analysis**

Statistical analysis was carried out using corrected Chi-squared test. *P* values < 0.05 were considered statistically significant.

**Results**

Using outer membrane proteins and whole cell antigens, 94 (82%) and 96 (84%) of the 115 gastric cancer patients and 80 (73%) and 84 (76%) of the 110 patients without gastric cancer were seropositive for anti-*H. pylori*. There were no differences between the seroprevalence of *H. pylori* in patients with gastric cancer and patients without.

Seven antigens were commonly recognized by sera from seropositive patients with gastric cancer and without gastrointestinal diseases (Table 1). Antibodies to CagA (120 kDa) antigen were detected using outer membrane protein antigen in 85 (90%) of the gastric cancer patients and in 67 (84%) of patients with diseases unrelated to *H. pylori* status. In all, 87 (91%) gastric cancer patients and 70 (83%) subjects without gastric cancer were seropositive for anti-CagA antibody using whole cell antigen. No association between the prevalence of antibody to the CagA antigen and gastric cancer was found in Lithuanian subjects. Of the 94 gastric cancer cases, 80 (85.1%) were seropositive for anti-
VacA antibody compared with 61 of 80 (76%) asymptomatic subjects. Antibodies to VacA antigen were prevalent in both groups, regardless of the presence of gastroduodenal disease. There was no difference between the frequency of the VacA antigen in gastric cancer patients and in patients without gastrointestinal pathology. IgG antibodies to the 94-kDa protein were detected significantly more frequently (P < 0.001) in gastric cancer patients (54%) than in patients without gastric cancer (20%). Serum IgG antibodies to the 30 kDa (UreA) antigen were found in 65% of gastric cancer patients and in 44% patients without gastrointestinal diseases. The difference between these two groups was statistically significant (P < 0.01).

Looking at the 33, 29 and 26 kDa outer membrane proteins, no significant differences were found between gastric cancer patients (40%, 16%, 22%) and patients without gastric cancer (51%, 20%, 19%), respectively (Table 1).

Specific IgG antibodies to both (VacA+ and CagA−) proteins were detected in 88% of patients with gastric cancer and in 71% of patients without gastric cancer. The difference was statistically significant (P < 0.01). The antibody response to CagA+/VacA− or CagA+/VacA+ was observed significantly more often (P < 0.001) in patients without gastrointestinal diseases (26%) than in patients with gastric cancer (5.3%) (Table 2).

### Discussion

Gastric cancer is one of the most frequently diagnosed malignancies in the world and is known to be more prevalent in populations with poor socioeconomic conditions. In Lithuania, 36.7 males and 21.1 females per 100 000 persons developed gastric cancer in 2004. According to the Lithuanian Cancer Register, gastric cancer remains the second leading cause of cancer death (30.4 deaths in males and 17.8 in females per 100 000 people) in Lithuania, as well as worldwide.

A number of studies have shown that *H. pylori* carriage is associated with adenocarcinoma of the stomach. Our previous study indicated a high prevalence of *H. pylori* infection in Lithuania when serum IgG antibody response to *H. pylori* was analyzed in patients with and without gastric cancer using enzyme-linked immunosorbent assay (ELISA) (Janulaityte-Gunther et al., 2005). In that study, using low molecular mass *H. pylori* antigen, a higher *H. pylori* seropositivity was detected in gastric cancer patients compared with asymptomatic patients (77% vs. 57%, P < 0.05). No significant difference was found using whole cell antigen or outer membrane proteins for the ELISA in a previous study, which is in accordance with the immunoblot results in the present study. The high prevalence of *H. pylori* in patients with gastric carcinoma is in accordance with studies from Italy (Palestro et al., 2005), Sweden (Held et al., 2004), Turkey (Abasiyanik et al., 2002) and Estonia (Vorobjova et al., 2006).

There is evidence that cagA-positive strains cause more serious infections (Iaquinto et al., 2000), achieve higher bacterial density on the gastric mucosa, and cause more inflammation than cagA-negative strains (Mitchell et al., 1996). In this study population we did not observe a tight association between gastric adenocarcinoma and the presence of IgG antibodies directed against CagA. Serum anti-CagA seropositivity was slightly, but not significantly, greater in patients with gastric cancer than in asymptomatic controls. The high CagA+ IgG seropositivity in Lithuanian people both with and without gastric cancer is comparable with the high prevalence reported for Turkish people, in whom a similar seroprevalence of CagA+ *H. pylori* in gastric cancer patients and patients without gastrointestinal symptoms was found (Abasiyanik et al., 2002). Sokic-Milutinovic et al. (2004) also reported a high (94%) anti-CagA seropositivity in patients from Serbia and Montenegro, with no

### Table 1. Distribution of the antibody reactivity to major Helicobacter pylori antigens in gastric cancer patients and the control group that have antibodies to *Helicobacter pylori* outer membrane proteins

<table>
<thead>
<tr>
<th>Immunoreactive band (kDa)</th>
<th>Patients with gastric cancer</th>
<th>Patients without gastric cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 94</td>
<td>n = 80</td>
</tr>
<tr>
<td>120 (CagA)</td>
<td>85 (90%)</td>
<td>67 (84%)</td>
</tr>
<tr>
<td>94</td>
<td>51 (54%)</td>
<td>16 (20%)</td>
</tr>
<tr>
<td>85 (VacA)</td>
<td>80 (85%)</td>
<td>61 (76%)</td>
</tr>
<tr>
<td>33</td>
<td>38 (40%)</td>
<td>41 (51%)</td>
</tr>
<tr>
<td>30 (UreA)</td>
<td>61 (65%)</td>
<td>35 (44%)</td>
</tr>
<tr>
<td>29</td>
<td>15 (16%)</td>
<td>16 (20%)</td>
</tr>
<tr>
<td>26</td>
<td>21 (22%)</td>
<td>15 (19%)</td>
</tr>
</tbody>
</table>

*Statistically significant difference (P < 0.001) between gastric cancer and controls.

### Table 2. Distribution of antibody responses to CagA and VacA presented as combinations of CagA/VacA in gastric cancer patients and the control group that have antibodies to *Helicobacter pylori* outer membrane proteins

<table>
<thead>
<tr>
<th>Helicobacter pylori Strain Type</th>
<th>Patients with gastric cancer</th>
<th>Patients without gastric cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td>CagA+/VacA−</td>
<td>83 (88%)</td>
<td>57 (71%)</td>
</tr>
<tr>
<td>CagA+/VacA+</td>
<td>6 (6.4%)</td>
<td>3 (3.8%)</td>
</tr>
<tr>
<td>CagA+/VacA− or CagA+/VacA+</td>
<td>5 (5.3%)</td>
<td>21 (26%)</td>
</tr>
</tbody>
</table>

*Statistically significant difference (P < 0.01) between patients with and patients without gastric cancer.

Statistically significant difference (P < 0.01) between patients without and patients with gastric cancer.
difference between those with gastroduodenal diseases and nonulcer dyspepsia. These data suggest that antibodies to CagA<sup>+</sup> are not a useful marker for the discrimination of *H. pylori* strains with respect to gastric cancer due to the high prevalence in the Lithuanian population.

In regard to anti-VacA antibodies, the data in the literature are more inconsistent. Although patients with gastrointestinal diseases are more frequently colonized by cytotoxicogenic *H. pylori* strain, the presence of anti-VacA antibodies is not consistently observed more frequently in these patients than in those without gastrointestinal pathology. There was no significant difference in the prevalence of antibodies to VacA in patients with gastric cancer (85%) and the group of patients without gastrointestinal diseases (76%) in the present study, in agreement with a previous study made in Taiwan (Kuo et al., 2003). Recently, Sezikli et al. (2006) demonstrated that serum IgG anti-VacA antibodies were present more frequently in patients with gastric cancer than in asymptomatic patients (86% vs. 23%), in agreement with the report by Sokic-Milutinovic et al. (2004), who showed a close association of VacA-positive *H. pylori* strain and gastrointestinal pathology, particularly with peptic ulcer disease.

Strains that possess both CagA and VacA are believed to be more pathogenic than strains lacking either CagA or VacA, or both, as shown in a study done in Turkey (Sezikli et al., 2006), where the CagA<sup>+</sup>/VacA<sup>+</sup> phenotype was more frequent in patients with duodenal ulcer, gastric ulcer, and gastric cancer than in asymptomatic patients (86% vs. 20%) (<i>P</i> < 0.01). In the present study we found that the CagA<sup>+</sup>/VacA<sup>+</sup> *H. pylori* strain is common in Lithuania and that there is a significantly positive association between the presence of anti-CagA and anti-VacA antibodies and gastric cancer in our region. These results disagree with Maeda et al.’s findings in Japan (Maeda et al., 1998), where CagA<sup>+</sup>/VacA<sup>+</sup> *H. pylori* infection is very common irrespective of the gastroduodenal status of the host. In contrast, the mixed (CagA<sup>+</sup>/VacA<sup>+</sup>, CagA<sup>-</sup>/VacA<sup>-</sup>) types of *H. pylori* strain are uncommon in our patients with gastric cancer, but are prevalent in asymptomatic Lithuanian patients. Therefore, additional studies are required to investigate the precise role of CagA andVacA.

Several studies have found that the seroprevalence of *H. pylori* decreases in patients with atrophic gastritis, generally found in patients with gastric cancer and older patients. The seroprevalence of CagA<sup>+</sup> *H. pylori* was significantly higher in younger (< 55 years) than in older patients with gastric cancer, but no differences in CagA<sup>-</sup> *H. pylori* seropositivity in the asymptomatic patients group, or in any *H. pylori* or other specific antigen examined in the two groups of patients were found in our study. This indicates that even though the immunoblot technique is more reliable than ELISA in patients with atrophic gastritis there may still be decreases in specific antibody levels caused by the atrophy.

A significant association has been found between antibody to the 30- and 45-kDa antigens and more serious gastroduodenal disease by Mitchell et al. (1996). A recent study made in Taiwan reported a significant association between the serological response to 19.5- and 26.5-kDa proteins and malignant outcome of *H. pylori* infection (Shiesh et al., 2000). The detection of IgG antibodies to 30-kDa band in our gastric cancer patients is also in accordance with our previous study (Janulaityte-Gunther, 2005), where a significant higher seroprevalence of *H. pylori* was found in patients with gastric cancer compared with the asymptomatic patient group using the low molecular mass antigen (15–30 kDa).

In summary, no significant differences between gastric cancer patients and patients without gastrointestinal symptoms were found using outer membrane proteins or whole cell antigens, considering antibodies to CagA and VacA, whereas significant differences were found looking at the combination of CagA and VacA as well as the 94- and 30-kDa bands. The prevalence of the 94- and 30-kDa bands is, however, too low to be of diagnostic or predictive value and the prevalence of CagA<sup>+</sup> and VacA<sup>+</sup> is too high to add a better discrimination even when all four are combined. However, it cannot be ruled out that these proteins are important, although not the only factor in gastric cancer development. The host and environmental factors may be more important predictors of disease outcome and hence to be considered as well.

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**References**


IgG antibodies to *H. pylori* in patients with gastric cancer


