GENOTYPES OF *HELIcobacter pylori* IN SYMPTOMATIC PATIENTS OF KARACHI, PAKISTAN

Muhammad Hanif1, Ajaz Rasool2, Parveen Zaidi1, Abid Hameed1 and Laeeque Ahmed3

1PCR and Molecular Biology lab, Karachi Institute of Radiotherapy and Nuclear Medicine,
2Laboratory of Molecular Genetics, Department of Microbiology, University of Karachi,
3Department of Gastroenterology, Liaquat National Hospital, Karachi.

ABSTRACT

*Helicobacter pylori* infections and gastroduodenal diseases have been reported several times in this region but there is no data regarding prevalence of *H. pylori* VacA and CagA genotypes in this part of the world. The objective of this study was to determine the prevalence of *H. pylori* genotypes in different gastroduodenal disease patients of Karachi.

Multiple biopsy samples were taken from 227 patients of peptic ulcer disease (PUD), chronic gastritis (CG) and/or abdominal discomfort (AD) in the gastroenterology departments from three different health institutions of Karachi and processed for rapid urease test, culture and histopathology. Positive isolates were further confirmed by PCR amplification of the phosphoglucomutase gene (glmM) and analyzed for the occurrence of CagA and VacA genotypes by PCR.

A significant association between *H. pylori* infection and gastroduodenal diseases was observed (p<0.001). Out of 227 biopsy samples 120 (52.8%) were confirmed positive for *H. pylori* in which 56 were that of PUD, 37 with CG, 19 with AD and 8 with gastric cancer (GCa). Overall 92% isolates from patients with PUD, CG, AD and GCa were CagA positive. Eighty five (70.8%) of 120 strains had VacA signal sequence genotype s1a, 19 (16%) had type s1b and 16 (13%) had type s2. The VacA middle region type m1 and m2 were detected in 78(65%) and 42 (35%) strains. The combinations s1a/m1 and s1a/m2 were found in 66(55%) and 19 (15.8%) respectively. The s1b/m1 and s1b/m2 were found in 12(10%) and 6(6.5%) strains respectively. The s2m2 combinations were identified in 15(12.5%) of 120 isolates. The s2m1 combination was not found in any strain. Forty eight (85.7%) of 56 patients with PUD, 25 (67.5%) of 37 patients with CG, 5 (26.3%) of 19 patients with AD and 7 (87.5%) of 8 with GCa harbored type s1a strains. Genotypes s1a/m1 with positive CagA are commonly associated with PUD, CG, AD and GCa.

Key-words: *Helicobacter pylori*, genotype, gastroduodenal disease, Karachi, Pakistan

INTRODUCTION

In developing countries, 70 to 90% of the population carries *H. pylori* and almost all of them acquire the infection before the age of 10 years (Taylor and Parsonnet., 1995). *H. pylori* colonize the gastric mucosa of humans and persist over decades if not treated. It causes chronic infection leading to gastritis. The infection is mostly asymptomatic, but a few individuals will develop severe diseases such as peptic ulcer disease (PUD), gastric adenocarcinoma, and mucosa-associated lymphoid tissue (MALT) lymphoma (Bickley et al., 1993; Marshall and Warren, 1984).

Several studies have been attempted to identify virulence markers in *H. pylori* allowing the disease outcome of an infection to be predicted, most of them were based on the analysis of VacA (vacuolating cytotoxin gene) and CagA (cytotoxin-associated gene) status (Van Doorn et al., 1998; Graham and Yamaoka., 1998; Yamaoka et al., 1999; Rudi et al., 2000; Atherton., 2000; Zheng et al., 2000).

The CagA gene is one of the 31 genes of a pathogenicity island called the cag pathogenicity island (Cag PAI) of about 40-kb DNA (Censini et al., 1996; Akopyants et al., 1998). The presence of CagA has been considered as a marker of the Cag PAI and has been associated with the more virulent *H. pylori* strains (Covacci et al., 1993). About 60% of *H. pylori* isolates possess the CagA gene, and nearly all of these express the CagA gene product (Covacci et al., 1993; Tummuru et al., 1993). The presence of the CagA gene in *H. pylori* strains increases the risk of developing peptic ulceration (Covacci et al., 1993) atrophic gastritis (Tummuru et al., 1993) and adenocarcinoma of the stomach (Blaser et al., 1995; Parsonnet et al., 1997). The CagA gene was successfully identified in patients with chronic gastritis and peptic ulcer disease (Tokumaru et al., 1999; Russo et al., 1999) as well as in patients with antral erosions and endoscopic features of normal mucosa (Russo et al., 1999). The CagA gene has also been detected in fecal samples of *H. pylori* infected, asymptomatic adult patients (Russo et al., 1999) and of children (Sicinchi et al., 2003).

Virulence factors also include the vacuolating cytotoxin (VacA). The VacA gene, which is present in all *H. pylori* strains, encodes a cytotoxin that damages epithelial cells by inducing the formation of vacuoles (Cover, TL., 1996) Within the VacA gene two variable segments have been identified the signal or s region and the middle or m region (Atherton et al., 1995). The signal sequence exists as s1 or s2 types and type s1 can be subtyped as s1a, s1b
and s1c (Yamaoka et al., 1999; Van Doorn et al., 1998). The middle region occurs as m1 or m2, and among type m1 strains, subtypes m1a and m1b can be distinguished (Atherton et al., 1995; Mukhopadhyay et al., 2000). It has been shown that the presence of CagA is strongly associated with VacA s1 genotype (Van Doorn et al., 1998). Whereas some studies have suggested that VacA s1 genotype, CagA and iceA1 are associated with the development of peptic ulcer disease (Van Doorn et al., 1998; Censini et al., 1996; Atherton et al., 1995), others have failed to confirm the association (Yamaoka et al., 1999; Graham et al., 1996). The discrepant results may be largely due to the fact that there are significant geographical differences in the genotypes among strains.

In the present study, H. pylori infection and prevalence of VacA and CagA genotypes among patient samples from three different health institutions of Karachi was determined and analyzed.

MATERIALS AND METHODS

Patients:
Two hundred and twenty seven patients under going upper gastro duodenal endoscopy for diagnosis and treatment purpose in the gastroenterology departments of three different health institutions of Karachi viz., Liaquat National Hospital (LNH), Pakistan Medical Research council (PMRC) and Jinnah Post Graduate Medical Centre (JPMC-Ward 7) were included in this study. The Institutional Review Board of KIRAN (Karachi Institute of Radiotherapy and Nuclear Medicine) Hospital and Pakistan Medical Research council (PMRC) approved the research protocol. All patients gave written consent for biopsy sampling.

Endoscopy, Biopsy sampling and Histopathology:
Four gastric biopsy specimens from antrum and corpus from each patient were collected for culture, histopathology and PCR. Biopsy specimens for culture and PCR were taken into screw caped bottle containing 0.9% saline and transported in the Microbiology and molecular biology Lab at KIRAN Hospital Karachi. The fourth biopsy specimen was directly used for genotyping by PCR. Biopsies were processed as previously described (Hanif et al., 2009).

Rapid Urease Test: One biopsy specimen from each sample was introduced with a sterile medium in to a semisolid 2% urea agar and incubated at room temperature. Results were recorded up to 4 h after inoculation (Deltenre et al., 1989).

Culture: Biopsies were processed as previously described (Hanif et al., 2009).

DNA extraction from biopsy specimens
Biopsies for DNA extractions were processed as previously described (Hanif et al., 2009).

DNA extraction from H. pylori cultures
Genomic DNA was extracted from each strain of H. pylori using commercially available kit (Gentra, PUREGENE USA) according to the manufacturer’s instructions and dissolved in distilled water and processed as previously described (Hanif et al., 2009).

H. pylori diagnostic PCR
The presences of H. pylori DNA was confirmed by PCR amplification of the phosphoglucosamine mutase gene, glmM and were processed as previously described (Hanif et al., 2009).

PCR amplification of CagA
PCR amplification of CagA was carried out using two primer sets named CagA1/CagA2 and CagA5/CagA2 (Pan et al., 1997). Primer sequences and their locations in the gene are shown in Table 1. 10 ng of DNA was used in a standard PCR mixture of 25 µl containing 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 3.0 mM MgCl2, and 0.1 mg of bovine serum albumin per ml. The incubation conditions with primer set 1 were as follows: 40 cycles of 1 min at 95°C, 1 min at 55°C, and 1.5 min at 72°C and final 5-min incubation at 72°C. When primer set 2 was used the number of cycles was 35 and the annealing temperature was 60°C. PCR products were analyzed by horizontal agarose (1.5%) gel electrophoresis with ethidium bromide staining. Negative and positive control amplifications were performed in every experiment.
Table 1. Primer sequences, their position in the gene and expected product sizes.

<table>
<thead>
<tr>
<th>Primer sequences for CagA1/CagA2 and CagA5/CagA2</th>
<th>Primer sequences for VacA Signal sequence variants</th>
<th>Primer sequences for vacA middle region variants</th>
</tr>
</thead>
<tbody>
<tr>
<td>CagA1 (5’-GATATAGCCACTACC ACCAC CG-3’) (1249 to 1270) (570 bp)</td>
<td>s1a 5’ GTCAGCATCACCACCGCAAC 3’ (866–1055) (190 bp)</td>
<td>m1 5’ GGTCAAAAATGCGGTCATGG 3’ (2741–3030) (290 bp)</td>
</tr>
<tr>
<td>CagA2 (5’-GGAAATCTTTAATCTCAGTT CGG-3’) (1797 to 1819)</td>
<td>5’ CTGCTTGAATGCGCAAAC 3’</td>
<td>5’ CCATTGGTACCTGTAGAAAC 3’</td>
</tr>
<tr>
<td>CagA5 (5’-GGCAATGGGT GTCCCTGGACGTAGGC-3’) (1495 to 1519) (324 bp)</td>
<td>s1b 5’ AGCGCCATAACCACCGAAG 3’ (869–1055) (187 bp)</td>
<td>m2 5’ GGAGCCCCAGGAAACATTG 3’ (976–1327) (352 bp)</td>
</tr>
<tr>
<td>CagA2 (5’-GGAAAC TCTTT AATCT CAGTT CGG-3’) (1797 to 1819)</td>
<td>5’ CTGCTTGAATGCGCAAAC 3’</td>
<td>5’ CATAACTAGCGGCTTGCAC 3’</td>
</tr>
</tbody>
</table>

Table 2. Distribution of H. pylori positive samples in various gastroduodenal diseases.

<table>
<thead>
<tr>
<th></th>
<th>Liaquat National Hospital (LNH)</th>
<th>Pakistan Medical Research council (PMRC)</th>
<th>Jinnah Post Graduate Medical Center (JPMC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of samples collected</td>
<td>H. pylori Positive</td>
<td>No. of samples collected</td>
<td>H. pylori Positive</td>
</tr>
<tr>
<td>Total number of samples</td>
<td>85</td>
<td>81</td>
<td>43</td>
</tr>
<tr>
<td>Peptic Ulcer Disease (PUD)</td>
<td>55 (64.7%)</td>
<td>43 (53%)</td>
<td>23 (57.5%)</td>
</tr>
<tr>
<td>Chronic gastritis (CG)</td>
<td>25 (71.4%)</td>
<td>20 (40%)</td>
<td>8 (40%)</td>
</tr>
<tr>
<td>Abdominal discomfort (AD)</td>
<td>21 (70%)</td>
<td>8 (40%)</td>
<td>6 (60%)</td>
</tr>
<tr>
<td>Gastric Carcinoma (Ca)</td>
<td>20 (45%)</td>
<td>9 (45%)</td>
<td>6 (60%)</td>
</tr>
</tbody>
</table>

PCR amplification of VacA

The oligonucleotides forward and reverse primers were synthesized from IDT, USA are listed in Table 1. PCR was performed in 50 µl reaction mixtures containing 100 ng of genomic DNA, 250 nM of each primer, 1X reaction buffer, 1.5 mM MgCl2, 1 U of Taq-DNA polymerase and distilled water in a PCR system (Thermo Hybaid, USA). After denaturation at 94°C for 5 min, amplification was carried out for 27 cycles at 94°C for 30 s, 52°C for 30 s, and 72°C for 30 s; the mixture was then cycled at 72°C for 7 min to complete the elongation step and was finally stored at 4°C (Atherton et al., 1995). For identification of the amplified products, 10 µl of the PCR mixture was analyzed...
by electrophoresis on 2% agarose gel (Serva, USA), stained with ethidium bromide 0.5 mg/ml and read under UV illumination.

Table 4. Number of patients, type of disease, numbers of *H. pylori* positive samples and *VacA* genes in various gastroduodenal diseases.

<table>
<thead>
<tr>
<th>No. of patients</th>
<th>Peptic ulcer Disease (PUD) 101(44.4%)</th>
<th>Chronic gastritis (CG) 71(31.2%)</th>
<th>Abdominal discomfort (AD) 40(17.6%)</th>
<th>Gastric cancer (GCa) 15(6.6%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>H. pylori</em> Positive</td>
<td>120</td>
<td>56(46.6%)</td>
<td>37(30.8%)</td>
<td>19(15.8%)</td>
</tr>
<tr>
<td><em>CagA</em> Positive</td>
<td>109</td>
<td>52(47.7%)</td>
<td>34(31.1%)</td>
<td>15(13.7%)</td>
</tr>
</tbody>
</table>

**VacA signal type**

<table>
<thead>
<tr>
<th>VacA s1a</th>
<th>85</th>
<th>48(56.4%)</th>
<th>25(29.4%)</th>
<th>5(5.8%)</th>
<th>7(8.2%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VacA s1b</td>
<td>19</td>
<td>6(31.5%)</td>
<td>9(47.3%)</td>
<td>3(15.7%)</td>
<td>1(5.2%)</td>
</tr>
<tr>
<td>VacA s2</td>
<td>16</td>
<td>2(12.5%)</td>
<td>3(18.7%)</td>
<td>11(68.7%)</td>
<td>0(0%)</td>
</tr>
</tbody>
</table>

**VacA middle region**

<table>
<thead>
<tr>
<th>VacA m1</th>
<th>78</th>
<th>46(58.9%)</th>
<th>24(30.7%)</th>
<th>0(0%)</th>
<th>8(10.2%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VacA m2</td>
<td>42</td>
<td>11(26.1%)</td>
<td>14(33.3%)</td>
<td>17(40.4%)</td>
<td>0(0%)</td>
</tr>
</tbody>
</table>

Table 4. Six possible combinations of *VacA* homologue containing signal sequence and middle region of *VacA* alleles and their relationship with *CagA* in various gastroduodenal diseases.

<table>
<thead>
<tr>
<th></th>
<th>Peptic ulcer Disease (PUD) CagA+</th>
<th>Chronic gastritis (CG) CagA+</th>
<th>Abdominal discomfort (AD) CagA+</th>
<th>Gastric cancer (GCa) CagA+</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total</strong></td>
<td>56(46.6%)</td>
<td>37(30.8%)</td>
<td>19(15.8%)</td>
<td>15</td>
</tr>
<tr>
<td><em>VacA s1a/m1</em></td>
<td>36(54.5%)</td>
<td>20(30.3%)</td>
<td>19</td>
<td>4(6%)</td>
</tr>
<tr>
<td><em>VacA s1a/m2</em></td>
<td>12(63%)</td>
<td>5(26.3%)</td>
<td>12</td>
<td>2(16.6%)</td>
</tr>
<tr>
<td><em>VacA s1b/m1</em></td>
<td>4(33.3%)</td>
<td>1(41.6%)</td>
<td>7</td>
<td>2(16.6%)</td>
</tr>
<tr>
<td><em>VacA s1b/m2</em></td>
<td>2(25%)</td>
<td>4(50%)</td>
<td>10(12.5%)</td>
<td>1</td>
</tr>
<tr>
<td><em>VacA s2/m1</em></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>VacA s2/m2</em></td>
<td>2(13.3%)</td>
<td>3(20%)</td>
<td>10(66.6%)</td>
<td>3</td>
</tr>
</tbody>
</table>
Fig 1. Agarose 1.5% gel electrophoresis of PCR products for *H. pylori* genotyping of representative strains. A. ureC gene; B. Cag A gene; C. VacA s1A gene; D. VacA s1B; E. VacA s2; F. VacA m1 and G. VacA m2 gene subtypes.
RESULTS

Patients consisted of 113 females and 114 males (Mean age 44 years; age range 18 to 78 years). Out of 113 females 58 were H. pylori positive and out of 114 males 62 were H. pylori positive. A total of 227 biopsy samples were collected and patient’s history record revealed peptic ulcer in 101 patients, chronic gastritis in 71 patients, and abdominal discomfort in 40 patients and gastric carcinoma in 15 patients (Table 2). Only 120 (52.8%) were confirmed H. pylori positive by PCR (band size of 294 bp) (fig. 1a) and other methods. Among 120 infected patients 56 were that of peptic ulcer disease (PUD), 37 with chronic gastritis (CG), 19 with abdominal discomfort (AD) and 8 with gastric cancer (GCa).

Prevalence of CagA gene

A total of 109 patient samples out of 120 (90.8%) were found CagA positive, out of them 52 (92.8%) patients having PUD, 34 (91.8%) have CG, 15 (78.9%) have AD and 8 (100%) have GCa (Table 2). The CagA gene was detected using specific set of CagA1/CagA2 primer having 98% sensitivity (band size of 324bp) and primer set CagA5/CagA2 having 100% sensitivity (band size of 570 bp) (Fig. 1b).

Prevalence of VacA gene

The VacA gene was detected in all 120 H. pylori isolates using primers s1a, s1b, s2, m1 and m2 (Table 1). Eighty-five (70.8%) of 120 strains yielded 190 bp product representing VacA signal sequence genotype s1a, 19 (16%) yielded the 187 bp product representing genotype s1b and 16 (13%) yielded 199 bp product representing genotype s2 (Figure 1c, 1d, 1e).

DNA sequences of all 120 H. pylori strains amplified either by the primers m1 representing type m1 (290 bp), or by the primers m2 specific for type m2 (352 bp) (Table 1, Fig 1f). Seventy eight (65%) isolates were classified as type m1 and forty two (35%) were classified as type m2 (Table 3).

Association of particular VacA genotypes with the occurrence of different gastrointestinal diseases was also determined. Forty eight (85.7%) of 56 patients with PUD, 25 (67.5%) of 37 patients with CG, 5 (26.3%) of 19 patients with AD and 7 (87.5%) of 8 with GCa harbored type s1a strains. Type s1b strains were found in only 6 (10.7%) of 56 patients with PUD compared with 9 (24.3%) of 37 subjects with CG, 3 (15.7%) of 19 with AD and 1 (14.2%) of 8 with GCa. Similarly only two strains were isolated from patients with PUD type s2; three strains were isolated from patients with CG type s2 and 11 (57.8%) of 19 with AD, while no strain was isolated with type s2 in patients of GCa (Table 3).

Five of six possible combinations of VacA homologue containing signal sequence and middle region (s1a/m1, s1a/m2, s1b/m1, s1b/m2, and s2/m2) were estimated. The s1a/m1 and s1a/m2 combinations were found in 66 (55%) and 19 (15.8%) respectively. The s1b/m1 and s1b/m2 were found in 12 (10%) and 8 (6.6%) strains respectively. The s2/m2 combinations were identified in 15 (12.5%) of 120 isolates. The s2/m1 combination was not found in any strain (Table 4).

Distribution of combinations of VacA homologue containing signal sequence and middle region (s1a/m1, s1a/m2, s1b/m1, s1b/m2, and s2/m2) with the occurrence of different gastrointestinal diseases was also determined (Table 4). The s1a/m1 was predominant combination found in 36 (64%) of 56 patients with PUD, 20 (54%) of 37 patients with CG, 4 (21%) with AD and 7 (85.7%) with GCa respectively (Table 4).

Association of VacA genotypes with CagA gene

When the VacA signal sequence type was compared with CagA status, 79 (92.9%) of 85 VacA type s1a strains, 16 (84.2%) of 19 VacA type s1b strains and 14 (87.5%) of 16 VacA type s2 strains were CagA positive (Table 4). This result suggests the close association between CagA status and the s1a signal sequence. A significant association was also found between VacA mid-region typing and CagA status. Among the 78 m1-type strains 71 (91%) were positive for CagA gene and 38 (90.4%) of 42 VacA type m2 strains were CagA positive.

DISCUSSION

In this study, we have investigated dyspeptic patients having complaints of peptic ulcer, chronic gastritis and/or abdominal discomfort in the gastroenterology departments from three different health institutions of Karachi by endoscopy and gastric biopsies for H. pylori infection and their molecular characterization. The present study confirmed the high prevalence of H. pylori in symptomatic patients of Karachi previously reported by other authors as well (Hanif et al., 2009; Abbas et al., 1998; Kazi et al., 1990). We found that ureC PCR was at least as sensitive as culture for detecting H. pylori infection. The rapid-urease test and histological examinations of biopsy specimens
detected *H. pylori* infection in slightly fewer patients than did culture but they still yielded good specificity. Overall, our results are in good agreement with other data for comparisons of different routine tests for *H. pylori* in which Giemsa staining of histological sections (Deltenre et al., 1989) or the agreement among all the tests was taken as the gold standard (Fabre et al., 1989). Overall, PCR assays detected *H. pylori* infection in a higher percentage of patients.

In present study *H. pylori* isolates were characterized on the basis of VacA (vacuolating cytotoxin gene) and CagA (cytotoxin-associated gene). Overall 90 % isolates were found to have CagA gene (CagA +) in which 92% have peptic ulcer disease, 91% have gastritis, 79% have abdominal discomfort and 100% have GI cancer carried the CagA gene. A high percentage of infections with CagA positive isolates were similarly reported from India, China, Japan, Korea, and Singapore (Yamaoka et al., 1999; Zheng et al., 2000; Mukhopadhyay et al., 2000; Pan et al., 1997; Hua et al., 2000). Conversely, reports from Germany, Netherlands and Italy indicated lower overall infection with CagA positive isolates and suggested that gastric colonization by such isolates was associated with a higher risk of ulcer development (Van Doorn et al., 1998; Rudi et al., 2000; Carratotil et al., 2000). These inconsistencies between reports imply that CagA may or may not be a universal virulence marker.

The VacA signal sequence genotypes (s1a, s1b, and s2) were identified in all *H. pylori* strains. For determination of the VacA mid-region genotypes, oligonucleotide primers m1 and m2 was allowed to characterize 100% of the *H. pylori* strains isolated from patients of three different health institutions of Karachi. *H. pylori* strains with the VacA signal sequence type s1a were predominant, whereas strains with type s1b and s2 rarely occurred, although in contrast with reports on *H. pylori* isolates from other countries (Van Doorn et al., 1998; Yamaoka et al., 1999). The equally important finding complements the strong association between peptic ulcer disease and VacA type s1a strains that *VacA* type s2 strains are rarely associated with peptic ulceration

The VacA genotypes of our *H. pylori* isolates were identical to those reported from India (Mukhopadhyay et al., 2000). However, in India, 7.2% strains had mixed s1 and s2 genotypes. Multiple VacA s genotypes, frequently reported in other countries (Van Doorn et al., 1998), were totally absent in our study.

We found that the s1a/m1 family of VacA alleles of *H. pylori* is predominant in this study, which differ from the findings from other Asian countries (Sicinchi et al., 2003) where the genotypes s1a/m2 are more common. The VacA genotype s2/m1 was not identified in our study, which is in consistent with the pioneering study of Atherton, and co-workers (Atherton et al., 1995) who also failed to detect the s2/m1 genotype, suggesting that strains with this genotype suffer from a selective disadvantage or are not viable. However, VacA signal sequence type s1, particularly s1a, and genotype s1a/m1 appeared to occur more frequently in our study.

A strong genetic association between of CagA and VacA signal sequence type s1a were found in this study, a significant association was also found between VacA mid-region typing and CagA status. Why two genetic elements without any physical linkage on the *H. pylori* chromosome should be so closely associated, is not clear. One hypothesis is that there are two clonal *H. pylori* populations (*VacA* s1/CagA and *VacA* s2/CagA). Another possibility is that there may be a functional linkage, whereby a selective advantage conferred by each gene product is manifested only in the presence of the other.

It is clear that *H. pylori* strains with the VacA signal sequence type s1a and middle region allele m1 were predominant in all the samples collected from three institutions of Karachi. All combinations of these *VacA* alleles occurred, with the exception of s2/m1. Type s1a strains were associated with ulcer and the presence of the CagA gene. The findings suggest that CagA + *H. pylori* strains with the VacA genotype s1a increase the risk for peptic ulcer diseases. Thus, VacA genotyping may allow identification of infected subjects at different risk levels.

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