

# Association of peptic ulcer with increased expression of Lewis antigens but not *cagA*, *iceA*, and *vacA* in *Helicobacter pylori* isolates in an Asian population

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## Abstract

**Background**—Studies in Western populations suggest that *cagA*, *iceA*, and *vacA* gene status in *Helicobacter pylori* isolates is associated with increased virulence and peptic ulcer disease.

**Aim**—To investigate the relationship between peptic ulcer and expression of Lewis (Le) antigens as well as *cagA*, *iceA*, and *vacA* in *H pylori* isolates in Singapore.

**Methods**—Expression of Le antigens in *H pylori* isolates obtained from patients with dyspepsia was measured by enzyme linked immunosorbent assay. The *cagA*, *iceA*, and *vacA* status was determined by polymerase chain reaction.

**Results**—Of 108 *H pylori* isolates, 103 (95.4%) expressed Le<sup>x</sup> and/or Le<sup>y</sup>, while Le<sup>a</sup> and Le<sup>b</sup> were expressed in 23 (21.3%) and 47 (43.5%) isolates, respectively. Expression of two or more Le antigens (Le<sup>x</sup>, Le<sup>y</sup>, Le<sup>a</sup>, or Le<sup>b</sup>) was significantly higher in *H pylori* isolated from ulcer patients than in non-ulcer patients (89.6% *v* 73.2%, *p*=0.035). There were no significant differences in the prevalence of *cagA* or *iceA1* in *H pylori* isolates from peptic ulcer and non-ulcer patients (86.6% *v* 90.2% for *cagA*; 70.1% *v* 68.3% for *iceA1*), and no association of peptic ulcer with any specific *vacA* genotype.

**Conclusions**—The present study indicates that peptic ulcer disease is associated with increased expression of Lewis antigens but not *cagA*, *iceA*, or *vacA* genotype in *H pylori* isolates in our population. This suggests that *cagA*, *iceA*, and *vacA* are not universal virulence markers, and that host-pathogen interactions are important in determining clinical outcome.

(Gut 2000;47:18-22)

Keywords: Lewis blood group antigens; *cagA*; *iceA*; *vacA*; *Helicobacter pylori*; peptic ulcer

*Helicobacter pylori* is the major aetiological agent of chronic active gastritis and is generally accepted as having a causative role in the pathogenesis of peptic ulcer (PU) disease. *H pylori* infection has also been aetiologically linked to the development of gastric carcinoma.<sup>1,2</sup> It is estimated that more than 50% of the world's population are infected with *H pylori*. However, only a minority of *H pylori*

infected subjects develop PU or gastric cancer. The reasons for this are not well understood.

Vacuolating cytotoxin gene (*vacA*) s1a genotype and the cytotoxin associated gene (*cagA*) have been demonstrated to be related to the virulence of *H pylori* infection and the development of peptic ulcer.<sup>3,4</sup> However, there are also reports to the contrary.<sup>5-7</sup> A novel gene *iceA* (induced by contact with epithelium gene) has been reported and two allelic variants of the gene (*iceA1* and *iceA2*) described.<sup>8</sup> Studies based on Western populations suggested that *iceA1* is associated with PU.<sup>9,10</sup> Recent studies showed that the lipopolysaccharides (LPS) of most *H pylori* isolates express Lewis<sup>x</sup> (Le<sup>x</sup>) and/or Le<sup>y</sup> blood group antigens,<sup>11</sup> and these antigens are also expressed on human gastric mucosa.<sup>12</sup> It is postulated that this molecular mimicry may play a role in the pathogenesis of *H pylori* infections.<sup>13</sup> Peptic ulcer disease has been suggested to be associated with *H pylori* expression of Le<sup>x</sup>/Le<sup>y</sup>, and an association between *cagA* gene and expression of Le<sup>x</sup>/Le<sup>y</sup> has also been reported.<sup>14</sup> Expression of Le antigens and the prevalence of *iceA* have not been fully investigated in Asian countries where the prevalence of the *cagA* gene is high regardless of the presence of the disease.<sup>15</sup> *H pylori* strains may differ in various geographical regions<sup>7</sup> and studies in different populations may clarify the importance and universality of putative virulence factors. In the present study expression of Le antigens and the prevalence of *cagA* as well as *iceA* and *vacA* were investigated in 108 *H pylori* isolates in Singapore.

## Materials and methods

### PATIENTS AND *H PYLORI* ISOLATES

*H pylori* strains were isolated from the gastric biopsies of 108 patients undergoing upper gastrointestinal endoscopy for dyspepsia at the National University Hospital, Singapore. Informed consent was obtained from all patients for gastroscopy and biopsies. All patients included in the study were *H pylori* positive as assessed using the rapid urease test and culture. The patient population comprised 88 Chinese, 13 Indians, and seven Malays. Of these, 67 were males and 41 females. Mean age

**Abbreviations used in this paper:** *cagA*, cytotoxin associated gene; *iceA*, induced by contact with epithelium gene; *vacA*, vacuolating cytotoxin gene; Le, Lewis blood group antigen; PCR, polymerase chain reaction; PU, peptic ulcer; LPS, lipopolysaccharides; NUD, non-ulcer dyspepsia; OD, optical density.

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Accepted for publication  
11 January 2000

Table 1 Polymerase chain reaction (PCR) for amplification of *H pylori* *cagA*, *iceA*, and *vacA* genes

Region	Primer	Nucleotide sequence (5'→3')	Size of PCR product (bp)	Reference
<i>cagA</i>	<i>cagA</i> -F	AATACACCAACGCCTCCAAG	400	22
	<i>cagA</i> -R	TTGTTGCCGCTTTTGCTCTC		
<i>iceA1</i>	<i>iceA1</i> -F	GTGTTTTTAACCAAAGTATC	246	9
	<i>iceA1</i> -R	CTATAGCCAGTCTCTTTGCA		
<i>iceA2</i>	<i>iceA2</i> -F	GTTGGGTATATCACAATTTAT	229 or 334	9
	<i>iceA2</i> -R	TTGCCCTATTTTCTAGTAGGT		
<i>m1</i>	<i>m1</i> -F	GGTCAAATGCCGTCATGG	290	23
	<i>m1</i> -R	CCATTGGTACCTGTAGAAAC		
<i>m2</i>	<i>m2</i> -F	GGAGCCCAGGAAACATTTG	352	23
	<i>m2</i> -R	CATAACTAGCGCCTTGCCAC		
<i>m1T</i>	<i>m1T</i> -F	GGCCACAATGCAGTCATGG	290	6
	<i>m1T</i> -R	CTCTTAGTGCCTAAAGAAACA		
<i>m1Tm2</i>	<i>m1T</i> -F	GGCCACAATGCAGTCATGG	300	6
	<i>m2</i> -R	CATAACTAGCGCCTTGCCAC		
<i>s1a</i>	<i>s1a</i> -F	GTCAGCATCACACCGCAAC	190	23
	<i>s</i> -R	CTGCTTGAATGGGCCAAAC		
<i>s1b</i>	<i>s1b</i> -F	AGCGCCATACCGCAAGAG	187	23
	<i>s</i> -R	CTGCTTGAATGGGCCAAAC		
<i>s2</i>	<i>s2</i> -F	GCTTAACACGCAATGATCC	199	23
	<i>s</i> -R	CTGCTTGAATGGGCCAAAC		

was 46 years (16–78). Based on history and endoscopic examination, patients were classified into the following groups: duodenal ulcer (36), gastric ulcer (31), and non-ulcer dyspepsia (41). Non-ulcer dyspepsia (NUD) was defined as patients with neither a history of ulcer disease nor endoscopic evidence of ulcer disease. Upper gastrointestinal endoscopy was performed, and after completion of mucosal examination two biopsies were obtained from the gastric antrum within 2 cm of the pylorus. Our previous study<sup>16</sup> on multiple antrum biopsies showed a predominance of a single strain of *H pylori*. Bacteria isolated from gastric biopsies were grown on blood agar plates (blood agar base 2 supplemented with 5% horse blood) in a humidified incubator supplied with 5% CO<sub>2</sub> (Forma Scientific, USA) at 37°C for three days. Isolates were identified as *H pylori* based on Gram stain morphology and positive tests for urease, oxidase, and catalase.

#### ANTIGEN EXPRESSION IN LPS

Expression of various LPS epitopes in the *H pylori* isolates was measured using an enzyme linked immunosorbent assay (ELISA) as described by Simoons-Smit and colleagues.<sup>17</sup> The following murine monoclonal antibodies (Mabs) were used: Mab 54-1F6A, specific for Le<sup>x</sup><sup>18</sup>; Mab 1E52, specific for Le<sup>y</sup><sup>19</sup>; Mab 7Le, specific for Le<sup>a</sup> (Bioprobe, Netherlands); Mab 225Le, specific for Le<sup>b</sup> (Bioprobe); Mab 3-3A, specific for blood group A antigen<sup>17</sup>; and Mab 3E7, specific for blood group B antigen (Dako, USA). Optical density (OD) was read at 490 nm. An OD value of 0.2 was chosen as the cut off value because the sum of non-specific background binding values for Mabs and for the conjugate never exceeded an OD of 0.1. Synthetic protein linked Le antigens (that is,

Le<sup>x</sup>, Le<sup>y</sup>, Le<sup>a</sup> and Le<sup>b</sup> (IsoSep, Sweden)) were used as positive controls for the Mabs.

#### PCR IDENTIFICATION

##### Detection of *cagA* and *iceA*

DNA of each *H pylori* strain was isolated by chloroform-phenol extraction.<sup>20</sup> The polymerase chain reaction (PCR) was carried out in an amplification thermal cycler (Perkin-Elmer, USA) programmed according to the protocol of Zheng and colleagues.<sup>21</sup> The primers used for *cagA*,<sup>22</sup> *iceA1*, and *iceA2*<sup>9</sup> and the expected PCR fragment lengths are listed in table 1.

##### Genotyping of *vacA* alleles

The combination of primers described by Atherton and colleagues<sup>23</sup> and Wang and colleagues<sup>6</sup> were used to amplify the *vacA* gene fragments of all *H pylori* isolates. Typing of *vacA* for signal sequence region alleles and middle region alleles was carried out using the primers and methods as described by Atherton and colleagues<sup>23</sup> for *s1a*, *s1b*, *s2*, *m1*, or *m2* and Wang and colleagues<sup>6</sup> for *m1T* or *m1Tm2*. These seven pairs of primers and the expected PCR fragment lengths are listed in table 1.

#### STATISTICAL ANALYSIS

Frequencies were compared using two tailed Fisher's exact test (SPSS 9.0, Chicago, USA). OD values were expressed as mean (SD), and the distributions of ODs were compared by the Student's *t* test for comparison of means of independent samples. A *p* value <0.05 was considered statistically significant.

## Results

#### EXPRESSION OF LEWIS ANTIGENS IN CLINICAL ISOLATES

Of the 108 *H pylori* isolates, 86 were found to express both Le<sup>x</sup> and Le<sup>y</sup>, while 11 and six isolates expressed only Le<sup>x</sup> and Le<sup>y</sup>, respectively. Three isolates expressed only Le<sup>b</sup> while two other isolates were non-typeable. The study revealed that 23 (21.8%) isolates expressed Le<sup>a</sup> while 47 (43.5%) isolates expressed Le<sup>b</sup> (table 2). Only one isolate expressed blood group A antigen while none expressed blood group B antigen.

#### RELATIONSHIP BETWEEN Le EXPRESSION AND CLINICAL OUTCOME

Table 2 shows that of 67 isolates from patients with PU, 64 (95.5%) were positive for Le<sup>x</sup> and/or Le<sup>y</sup> antigens compared with 39 (95.1%) of 41 isolates from patients with NUD (*p*=1.000). Furthermore, the mean OD level of Le<sup>x</sup> or Le<sup>y</sup> expression was not significantly different between isolates from patients with or without ulcer disease (1.0150 (0.6515) *v*

Table 2 Relationship between *H pylori* expression of Le antigens, *cagA*, *iceA1*, *vacA*, and peptic ulcer

Lesion	Isolate	Le <sup>x</sup>	Le <sup>y</sup>	Le <sup>x</sup> and/or Le <sup>y</sup>	Le <sup>a</sup>	Le <sup>b</sup>	≥2 Le antigens*	<i>cagA</i>	<i>iceA1</i>	<i>iceA2</i>	<i>vacA s1a/m1T</i>
Peptic ulcer	67	61 (91.0)	58 (86.6)	64 (95.5)	18 (26.9)	34 (50.7)	60 (89.6)†	58 (86.6)	47 (70.1)	16 (23.9)	25 (37.3)
Non-ulcer	41	36 (87.8)	34 (82.9)	39 (95.1)	5 (12.2)	13 (31.7)	30 (73.2)	37 (90.2)	28 (68.3)	10 (24.4)	14 (34.1)
Total	108	97	92	103	23	47	90	95	75	26	39

Values are number (percentage).

\*Lewis antigens (Le<sup>x</sup>, Le<sup>y</sup>, Le<sup>a</sup>, or Le<sup>b</sup>).

†*p*<0.05 compared with non-ulcer group.

1.0813 (0.6496) for Le<sup>x</sup> (p=0.608); 1.3549 (1.1200) v 1.3774 (1.1917) for Le<sup>y</sup> (p=0.890)). Sixty (89.6%) of 67 isolates from PU patients expressed two or more Le antigens (Le<sup>x</sup>, Le<sup>y</sup>, Le<sup>a</sup>, or Le<sup>b</sup>) compared with 30 (73.2%) of 41 isolates from NUD patients (p=0.035). Furthermore, 32/67 (47.8%) isolates from PU patients expressed three or more Le antigens compared with 11/41 (26.8%) isolates from NUD patients (p=0.043).

Levels of expression of Le<sup>a</sup> and Le<sup>b</sup> were not significantly different in the *H pylori* isolates from patients with peptic ulcer compared with those without ulcers (26.9% v 12.2% (p=0.091) for Le<sup>a</sup> and 50.7% v 31.7% (p=0.072) for Le<sup>b</sup>, respectively). The results also showed that Le<sup>x</sup> coexpressed with Le<sup>b</sup>.

#### RELATIONSHIP BETWEEN *cagA* STATUS AND CLINICAL OUTCOME

The *cagA* gene was positive in 95 (88%) of 108 *H pylori* isolates. As shown in table 2, the *cagA* gene was found in 58 (86.6%) of 67 PU isolates compared with 37 (90.2%) of 41 non-ulcer isolates (p=0.763). This shows that there was no significant difference for the *cagA* gene in *H pylori* isolates from PU and NUD patients.

Of the 95 *cagA* positive isolates, 91 (95.8%) expressed Le<sup>x</sup> and/or Le<sup>y</sup> antigens compared with 12 (92.3%) of 13 *cagA* negative isolates (p=0.480). Furthermore, the mean OD level of Le<sup>x</sup> or Le<sup>y</sup> expression was not significantly different between *cagA* positive and *cagA* negative isolates (1.0490 (0.6125) v 0.9862 (0.8928) for Le<sup>x</sup> (p=0.745); 1.2860 (1.0970) v 1.8615 (1.3687) for Le<sup>y</sup> (p=0.088)).

#### RELATIONSHIP BETWEEN *iceA* STATUS AND CLINICAL OUTCOME

Of 108 isolates, *iceA1* was positive in 75 isolates and *iceA2* was detected in 26 isolates. Four isolates were positive for both *iceA1* and *iceA2*, while three isolates did not yield either *iceA1* or *iceA2* fragments. There was no significant difference in the presence of *iceA1* in *H pylori* isolates from PU and NUD patients (70.1% v 68.3%; p=0.833). *iceA1* was not associated with *cagA* status (p=0.531). Similarly, there was no significant difference in the presence of *iceA2* in *H pylori* isolates from PU and NUD (23.9% v 24.4%; p=1.000).

Of 75 *iceA1* positive isolates, 72 (96.0%) expressed Le<sup>x</sup> and/or Le<sup>y</sup> antigens compared with 31 (93.9%) of 33 *iceA1* negative isolates (p=0.640). The mean OD level of Le<sup>x</sup> or Le<sup>y</sup> expression was not significantly different between *iceA1* positive and *iceA1* negative isolates (1.0106 (0.6242) v 1.0699 (0.7026) for Le<sup>x</sup> (p=0.663); 1.2570 (1.0831) v 1.4669 (1.2140) for Le<sup>y</sup> (p=0.373)).

#### RELATIONSHIP BETWEEN *vacA* GENOTYPE AND CLINICAL OUTCOME

Of 108 isolates, 107 were typed as s1a of the *vacA* genotype and the other isolate was typed as s2. Four *vacA* genotypes (s1a/m1T, s1a/m1Tm2, s1a/m2, and s2/m2) of *H pylori* isolates were identified (table 3), with the distribution as follows: 39 s1a/m1T, 4 s1a/m1Tm2, 64 s1a/m2, and 1 s2/m2. There was

Table 3 Relationship between *H pylori vacA* genotype and peptic ulcer

Lesion	Isolate	s1a/m1T	s1a/m1Tm2	s1a/m2	s2/m2
Peptic ulcer	67	25 (37.3)	3	38 (56.7)	1
Non-ulcer	41	14 (34.1)	1	26 (63.4)	0
Total	108	39	4	64	1

Values are number (percentage).

no significant difference between PU and NUD patients for infection by s1a/m1T genotype *H pylori* isolates (37.3% v 34.1%; p=0.837).

## Discussion

In this study we observed the occurrence of Le<sup>x</sup>, Le<sup>y</sup>, Le<sup>a</sup>, Le<sup>b</sup>, and blood group A antigen in *H pylori* isolates. Of 108 isolates, 106 (98.1%) were typeable with Mabs specific for Lewis and other blood group antigens. The two isolates which were non-typeable showed O side chain (data not shown), indicating the existence of other serotypes that were not reactive with the Mabs used as described by Simoons-Smit and colleagues.<sup>17</sup> One strain of *H pylori* expressed blood group A antigen which has so far been reported in one other *Helicobacter* species, *H mustelae*.<sup>24</sup> However, the low prevalence of this antigen in *H pylori* isolates suggests that it does not have an important role in the pathogenesis of gastric diseases. The chemical structures of Le<sup>x</sup>, Le<sup>y</sup>, and Le<sup>a</sup> of *H pylori* were elucidated earlier,<sup>25</sup> while the chemical structure of Le<sup>b</sup> has recently been determined.<sup>26</sup>

Le<sup>x</sup> and Le<sup>y</sup> were frequently encountered in our local *H pylori* isolates. Expression of Le<sup>x</sup> and Le<sup>y</sup> was similar to the finding in Canada<sup>27</sup> but was higher than the findings in the USA<sup>14</sup> and Europe.<sup>17</sup> Expression of Le<sup>a</sup> and Le<sup>b</sup> in *H pylori* isolates in our study was much higher than that found in the USA,<sup>14</sup> Netherlands,<sup>17</sup> and Canada.<sup>27</sup> Broadberry and Lin-Chu reported that the Le(a+ b+) phenotype is frequent in Chinese patients but rare or absent in Caucasians.<sup>28</sup> The relationship between Le antigen expression by *H pylori* and host phenotype is not clear.<sup>27, 29</sup> Our observation of higher expression of Le<sup>a</sup> and Le<sup>b</sup> in our population of predominantly Chinese patients lends support to the suggestion by Wirth and colleagues<sup>29</sup> that *H pylori* Le antigen expression is related to the host phenotype. However, Taylor and colleagues did not find such a correlation in their study.<sup>27</sup>

Wirth and colleagues<sup>14</sup> suggested that the risk of peptic ulcer increases with expression of Le antigens in *H pylori* isolates. They found that expression of Le<sup>x</sup> or Le<sup>y</sup> in *H pylori* isolates was significantly higher in PU than in NUD patients. It is important to note that only three of 96 subjects in their multicentre study were of Chinese origin. In contrast, in our predominantly Chinese patients (88/108 (81.5%)), we found equally high expression of Le<sup>x</sup> and Le<sup>y</sup> in *H pylori* isolates from both PU and NUD patients. Furthermore, the mean OD level of Le<sup>x</sup> or Le<sup>y</sup> expression was not significantly different between the two groups.

In this study we showed that the presence of two or more Le antigens (Le<sup>x</sup>, Le<sup>y</sup>, Le<sup>a</sup>, or Le<sup>b</sup>)

was significantly higher in *H. pylori* isolates from PU than from NUD patients. Expression of Le<sup>a</sup> and Le<sup>b</sup> antigen in *H. pylori* isolates from patients with PU was not significantly different from NUD patients.

As this was an exploratory study on the expression of Le antigens in *H. pylori* strains and indeed the first such study in our population, statistical analyses were performed on multiple parameters. We cannot exclude the possibility that some findings may be due to chance. However, the finding of higher expression of Le antigens in peptic ulcer associated *H. pylori* strains is novel in our population because it holds true on testing for  $\geq 2$  Le antigens and  $\geq 3$  Le antigens.

Several studies in Caucasian populations suggest an association between infection by *cagA* positive *H. pylori* and PU disease.<sup>30-31</sup> Our study indicates that *cagA* status is not predictive of gastroduodenal disease in the Singapore population, and adds to the growing evidence<sup>32-33</sup> that the *cagA* gene should not be regarded as a universal virulence marker of peptic ulcer disease.

An association of peptic ulcer and infection by *iceA1* positive *H. pylori* isolates was described in two previous studies of Western patients.<sup>9-10</sup> In accordance with van Doorn and colleagues,<sup>9</sup> we found that the presence of *iceA1* was independent of the *cagA* gene. Similar to *cagA* status, *iceA1* of *H. pylori* was not associated with PU risk in our population. This constitutes further evidence that distinct *H. pylori* genotypes circulate in Western and Asian countries.<sup>15</sup>

Four *vacA* genotypes (s1a/m1T, s1a/m1Tm2, s1a/m2, and s2/m2) of *H. pylori* isolates were found in the Singapore population. In the present study almost all of the *H. pylori* isolates were typed as s1a, which is similar to reports from China<sup>5</sup> and Taiwan.<sup>6</sup> The majority (95.4%) of *H. pylori* isolates in Singapore were typed as s1a/m1T or s1a/m2, suggesting that there is less mosaicism in *vacA* alleles of *H. pylori* in Singapore where the population is of mainly Chinese origin. The s1a/m1 genotype *H. pylori* that was reported to be associated with PU<sup>3</sup> was not detected in our population. In contrast with the finding by Wang and colleagues,<sup>6</sup> there was no association between infection of s1a/m1T *H. pylori* isolates and peptic ulcer in the present study.

The present study showed that peptic ulcer disease was not associated with *cagA* status or *iceA* or *vacA* genotypes but there was an association with increased expression of a combination of Le antigens. This suggests that the pathogenesis of *H. pylori* induced gastric diseases may be due to host-pathogen interactions rather than *H. pylori* itself. Expression of Le antigens may favour development of disease in the host by two mechanisms. Firstly, *H. pylori* strains expressing Le antigens may adapt more readily to a host possessing similar antigens.<sup>29</sup> Expression of host related antigens on the surface of bacteria could allow bacteria to elude elimination by the host immune response and thus facilitate persistence of infection in the host.<sup>34</sup> Chronicity of infection may lead to the

development of disease by increasing inflammation and promoting mucosa atrophy. Secondly, expression of host related antigens on *H. pylori* may increase the pathogenic potential of the bacterium via induction of autoantibodies that cross react with gastric mucosa.<sup>13</sup> However, it is noted that expression of Le antigens in *H. pylori* was also high in NUD patients, which indicates that additional unidentified factors also contribute to the pathogenesis of *H. pylori* associated peptic ulcer in our population.

The project was supported by the National University of Singapore (NUS) grant GR6431. The authors thank Dr BJ Appelmek (Amsterdam Free University, Netherlands) for generously providing all the Mabs except 3E7. We thank A/Prof JL Ding (NUS) for helpful discussion. ZPY is a Research Scholar of NUS.

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