

The significance of cagA and vacA subtypes of *Helicobacter pylori* in the pathogenesis of inflammation and peptic ulceration

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Abstract

Aims—To assess the significance of cagA and vacA subtypes of *Helicobacter pylori* in relation to inflammation and density of bacterial colonisation in vivo within a dyspeptic UK population.

Methods—Dyspeptic patients who were *Helicobacter pylori* positive had antral samples taken for histology and culture. Gastroduodenal pathology was noted. The grade of bacterial density and inflammation was assessed using the Sydney system. Bacterial DNA was extracted and the vacA alleles and the cagA gene typed using PCR.

Results—120 patients were studied. There was high rate of cagA positive strains in this population. Bacterial density did not correlate with the presence of peptic ulceration. There was a significant association between cagA positive strains and increased inflammation and bacterial density. The vacA s1 type independently correlated with extensive chronic inflammation but there was no association with bacterial density. The vacA m type did not correlate with extent of inflammation or bacterial density.

Conclusions—The results suggest that cagA is important in the pathogenesis of inflammation and peptic ulceration. These findings are in keeping with the hypothesis that cagA acts as a marker for a cag pathogenicity island which encodes several genes involved in inflammation. The vacA s1 allele correlates with inflammation independently of cagA, possibly through its enhanced ability to produce the vacuolating cytotoxin.

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associated gene (cagA)—have been identified as possible virulence determinants.

The vacA gene encodes a toxin that induces vacuolation in mammalian cell lines² and gastric epithelial cell damage and mucosal ulceration on intragastric administration to mice.³ The gene is present in all strains of *H pylori*, but toxin activity is expressed in only 50% of strains.⁴ Subgenotypes of vacA have been identified and specific vacA alleles correlate with production of toxin in vitro and with the presence of peptic ulcer disease.⁵

The cagA gene is present in approximately 60% of strains and encodes a 120–140 kDa protein, the role of which is unknown.⁶ The expression of cagA closely correlates with in vitro cytotoxin production and gastroduodenal pathology, but insertional mutagenesis of cagA fails to disrupt toxin expression.⁷ Although the function of cagA is unknown, it is recognised as a marker of enhanced virulence and it is thought to identify an island of genetic pathogenicity.⁸ This “cag pathogenicity island” is a 40 kb foreign DNA insertion, of uncertain origin and evolutionary age, which encodes several genes involved in the induction of proinflammatory cytokines.^{8,9} The island DNA is distinguished from that of the host by a different G+C content.⁸

Several investigators have noted a correlation between strains that possess cagA and the severity of gastric mucosal inflammation,^{10,11} but the patient groups have been small. More recently, work in the USA has shown a relation between specific vacA alleles and the extent of gastric inflammation.¹² The aim of this study was to evaluate the relation between the cagA status and vacA genotypes and the density of bacterial colonisation and the extent of gastric inflammation in a group of symptomatic patients in the United Kingdom.

Methods

PATIENT SELECTION AND SAMPLES

We recruited dyspeptic patients undergoing routine upper gastrointestinal endoscopy who were *H pylori* positive on rapid urease test or histology, or both. Patients treated with non-steroidal anti-inflammatory drugs or proton pump inhibitors within the previous two weeks, previous *H pylori* eradication treatment, and a past history of gastric surgery were excluded. Additional biopsies were taken from the antrum for culture and biopsy DNA preparation. Macroscopic evidence of gastrointestinal disease was noted at the time of the procedure.

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Helicobacter pylori is one of the commonest chronic bacterial infections of humans worldwide and invariably causes a chronic superficial gastritis. However, in a minority of individuals, infection results in the development of serious gastroduodenal pathology.¹ The reason for such a clinically diverse outcome of infection with *H pylori* remains uncertain but environmental, host, and bacterial factors are all thought to be important. The potential pathogenic difference between strains of *H pylori* may be responsible for the difference in clinical sequelae, and two genes—the vacuolating cytotoxin gene (vacA) and the cytotoxin

Table 1 Breakdown of the *vacA* genotypes and the *cagA* status in all strains, peptic ulcer disease (PUD), non-ulcer disease (NUD), and cancer (Ca) strains

<i>vacA</i> type	All strains		NUD		PUD		Ca
	<i>cagA</i> +	<i>cagA</i> -	<i>cagA</i> +	<i>cagA</i> -	<i>cagA</i> +	<i>cagA</i> -	<i>cagA</i> +
s1/m1	39	6	21	4	16	2	2
s1/m2	40	12	23	9	15	3	2
s2/m2	3	10	3	9	0	1	0
s1 only	8	1	4	0	3	1	1
m2 only	1	0	1	0	0	0	0
Total	91	29	52	22	34	7	5

HISTOLOGY

Antral samples obtained by sterile gastric biopsy forceps were placed on filter paper and fixed in formalin. Tissue was processed within 24 hours. Sections were stained with haematoxylin-eosin and with a modified Giemsa stain and were then examined by a single experienced consultant histopathologist, who was blinded to the infecting *H. pylori* strain type. Extent of bacterial density and grade of acute and chronic inflammation was assessed using the Sydney system.¹³ Grades 0 and 1 were grouped together and represented mild or low inflammation/density, and grades 2 and 3 were grouped together, representing moderate/severe.

H. PYLORI INFECTION

H. pylori infection was diagnosed by culture (96 strains) or by polymerase chain reaction (PCR) detection of *vacA* and *cagA* gene sequences directly from gastric biopsies (24 strains).

Bacteria were isolated from antral biopsy samples as described previously.¹⁴ For DNA preparation, isolates were grown on chocolate agar microaerobically, at 37°C for 48 hours, harvested into 500 µl of sterile phosphate buffered saline (PBS; pH 7.4), and washed once. The cells were suspended in 200 µl of digestion buffer (100 mM NaCl/10 mM Tris/1 mM EDTA, pH 8.0, containing 100 µg/ml lysozyme (Sigma)). Samples were incubated at room temperature for 15 minutes, and sodium dodecyl sulphate (SDS) was added to a final concentration of 1% (vol/vol). Bacterial cell lysis was completed by heating to 65°C, before addition of proteinase K (Sigma) to 25 µg/ml. Samples were incubated at 50°C for two hours. Protein was removed by phenol/chloroform extraction and DNA recovered by ethanol precipitation and resuspended in 100 µl of sterile nanopure water.

In a subset of patients *H. pylori* infection was detected by PCR directly from antral biopsies. Biopsies were suspended in 300 µl of digestion buffer containing 25 µg/ml of proteinase K and incubated at 37°C overnight. Protein was removed by phenol/chloroform extraction and DNA isolated by ethanol precipitation. The DNA was resuspended in 30 µl of sterile nanopure water.

POLYMERASE CHAIN REACTION

The *cagA* gene and *vacA* alleles were detected by PCR using primers and protocols from published reports. The *cagA* gene was detected using the primers F1 and B1 to amplify a 349 bp segment from the middle of the gene.⁶ The

amplification protocol used was as described by Owen *et al.*,¹⁵ with 40 cycles of 94°C for 30 seconds, 60°C for 60 seconds, and 72°C for 75 seconds. Bacterial isolates were tested for *vacA* genotype with five sets of primers as described by Atherton and coworkers⁵: two sets of primers for the mid-region of the gene (m1 and m2) and three sets of primers for the signal sequence region (s1a, s1b, and s2). Biopsy DNA samples were examined for the presence of *vacA* m1 and m2, and *vacA* s type, using a single set of universal s region primers to detect a product that could be differentiated as s1 (259 bp) or s2 (286 bp) on the basis of size. We used an amplification protocol of 95°C for 60 seconds, 56°C for 60 seconds, and 72°C for 60 seconds, for 40 cycles. PCR products were analysed by Tris-acetate-EDTA gel electrophoresis on 2% agarose gels, or 3% agarose gels for the universal s region primer set, and DNA was visualised by ethidium bromide staining and ultraviolet transillumination.

STATISTICS

The results were analysed using the χ^2 test with Yates continuity correction and the Fisher exact test.

Results

PATIENT GROUPS

One hundred and twenty dyspeptic patients (age range 19–87 years, M:F ratio 75:45), who were *H. pylori* positive on rapid urease test or histology or both, were successfully typed for *cagA* and the *vacA* alleles. Seventy four were non-ulcer dyspeptics (NUD), 41 had peptic ulcer disease, of whom 16 had gastric ulcers and 25 duodenal ulcers, and five had gastric carcinomas.

STRAIN *cagA* STATUS AND *vacA* GENOTYPES

Ninety one of 120 strains (76%) were *cagA* positive. The *vacA* genotypes of the strains were as follows: 45 s1/m1, 52 s1/m2, 13 s2/m2; one strain was non-typable for the *vacA* s region and nine strains were non-typable for the *vacA* m region. There were no strains of the type s2/m1 (table 1).

STRAIN TYPES AND PATHOLOGY

In patients with peptic ulcer disease, 34/41 (83%) were *cagA* positive and *vacA* s1 type. Only one strain was of the type s2/m2. Within this group 11/16 gastric ulcer strains were of the type *vacA* s1/m1 compared with 11/25 duodenal ulcer strains. Of the NUD strains, 52/74 were *cagA* positive (70%); 25 strains were s1/m1, 32 were type s1/m2, and 12 were type s2/m2. All five gastric cancer patients were *cagA* positive and *vacA* s1 (table 1).

BACTERIAL DENSITY IN ASSOCIATION WITH PEPTIC ULCER DISEASE

Bacterial density was low (grade 0–1) in 33/74 of NUD strains and 20/41 of the ulcer strains, showing there was no significant correlation between the density of bacterial colonisation and the presence of peptic ulceration ($p = 0.8$; table 2) Within the ulcer group, this lack of association was seen for both the duodenal and

Table 2 Bacterial density and peptic ulceration

	Grade of bacterial density	
	0-1	2-3
NUD strains	33	41
PUD strains	20	21

NUD, non-ulcer dyspeptic; PUD, peptic ulcer disease.

gastric ulcer strains (12/25 duodenal ulcer and 8/16 gastric ulcer strains had low levels of bacterial colonisation).

cagA STATUS, BACTERIAL DENSITY, AND INFLAMMATION

In patients infected with cagA positive strains there was a denser colonisation of *H. pylori* in the antrum ($p = 0.001$) and a significant increase in the inflammatory response when compared with patients infected with cagA negative strains (chronic, $p = 0.003$; acute, $p = 0.011$) (table 3).

In the peptic ulcer disease patients, 24/34 of the cagA positive strains were associated with severe acute inflammation compared to 1/7 of the cagA negative strains ($p = 0.009$). A similar statistically significant correlation was seen between cagA positive strains and bacterial density (21/34 cagA positive strains *v* 0/7 cagA negative strains were associated with a dense colonisation, $p = 0.003$).

In the NUD strains, there was a significant association between cagA positive strains, bacterial density (33/52 cagA positive *v* 8/22 cagA negative strains were grades 2-3; $p < 0.03$), and chronic inflammation (42/52 cagA positive

v 11/22 cagA negative strains were grades 2-3; $p < 0.009$).

VACA TYPE, BACTERIAL DENSITY, AND INFLAMMATION

Type s1 strains were associated with a marked chronic inflammatory infiltrate (grades 2-3). There was no correlation with acute inflammation or density of bacterial colonisation. When type s1 was analysed in relation to cagA positive and cagA negative strains the association with severe chronic inflammation persisted ($p = 0.035$), showing that the association was independent of cagA (table 4). There was a similar trend within the peptic ulcer disease strains.

The number of vacA s2 strains in this study population was very small (13) and thus we were unable to make a reliable comparison of the effect of types s1 and s2 on inflammation and bacterial density.

There was no association between type m1 or type m2 and grade of bacterial density or inflammation independent of cagA. Types m1 and m2 were compared in patients with cagA positive strains and no significant difference was shown between the mid-region sequences and the extent of inflammation or density of bacterial colonisation (table 5).

Discussion

The cagA gene acts as a genetic marker for an island of genes that are thought to be responsible for the pathogenic mechanisms of *H. pylori*.⁸ This cag island of pathogenicity encodes several genes—including cagA and the genes picA and picB—which are involved in the enhanced release of the cytokine interleukin 8, and subsequent gastric inflammation and ulceration.⁹ Strains with the type vacA s1/m1 (associated with in vitro cytotoxin production) and expression of cagA are labelled as type 1 strains and are expected to result in a more marked inflammatory response and ulceration.¹⁶

Potentially, type 1 strains induce mucosal damage directly through cytotoxin production and highly immunogenic proteins (in particular the cytotoxin and the 120-140 kDa product of cagA). It has also been suggested that cagA positive strains may cause extensive inflammation not only by inducing pro-inflammatory cytokines, but also by increased ability of the bacteria to colonise the gastric mucosa.¹⁷

In this study the expression of cagA was associated with the presence of peptic ulcer disease and infecting cagA positive strains were associated with denser colonisation of *H. pylori* and a more marked antral inflammatory response when compared to cagA negative strains. These findings, in conjunction with other studies,^{11, 17} emphasise that cagA is important in bacterial colonisation and in the pathogenesis of inflammation and peptic ulceration. Work by Atherton *et al* and Khulusi *et al* showed an association between density of bacterial colonisation in vivo and the presence of ulceration,^{17, 18} but we were unable to demonstrate a similar convincing relation in this study. It is important to acknowledge that

Table 3 cagA Status, grades of inflammation, and density of bacterial colonisation

	All strains					
	Bacterial density		Acute inflammation		Chronic inflammation	
	0-1	2-3	0-1	2-3	0-1	2-3
cagA+ Strains	34	57	42	49	15	76
cagA- Strains	21	8	21	8	13	16

0-1, mild inflammation/low bacterial density; 2-3, moderate to severe inflammation/bacterial density.

Table 4 Association of vacA s1 with bacterial density and inflammation overall and independently of the cagA status

	All strains					
	Bacterial density		Acute inflammation		Chronic inflammation	
	0-1	2-3	0-1	2-3	0-1	2-3
vacA s1	47	59	53	53	20	74
s1/cagA+	32	55	39	48	13	74
s1/cagA-	15	4	14	5	7	12

0-1, mild inflammation/low bacterial density; 2-3, moderate to severe inflammation/bacterial density.

Table 5 Association of vacA m types with grades of inflammation and bacterial density in cagA+ strains

	All strains					
	Bacterial density		Acute inflammation		Chronic inflammation	
	0-1	2-3	0-1	2-3	0-1	2-3
m1/cagA+	16	23	18	21	10	29
m2/cagA+	15	29	20	24	5	39

0-1, mild inflammation/low bacterial density; 2-3, moderate to severe inflammation/bacterial density.

differences between studies may reflect the difficulties in accurately assessing bacterial density in an infection which is known to be patchy in nature.

In our study population, there was no difference in the distribution of vacA types s1/m1 and s1/m2 in the NUD and the peptic ulcer disease groups. It is of interest that there was only one type s2 strain in the peptic ulcer disease group compared with 12 in the NUD group. We were unable to make any further comparison between type s1 and type s2 in terms of inflammation and density, as the number of s2 types was too small to reach statistical significance. However, there was a significant correlation between vacA s1 type and enhanced chronic inflammation, independent of the cagA status of the strain. This trend is in keeping with a study in the USA¹² and could be explained by the finding that greater levels of the vacuolating cytotoxin are produced by the vacA s1 type than by other vacA genotypes.⁵ Thus we would expect the s1 strains to cause extensive epithelial cell damage, inflammation, and potentially ulceration.

Analysis of the vacA mid-region showed there was no correlation with either bacterial density or enhanced inflammation. However, there was an interesting relation between gastroduodenal ulcers and the vacA mid-region in this study. The majority of the gastric ulcer strains were vacA m1 compared with the duodenal ulcer strains which showed a predominance of m2 types. The significance of this is uncertain and further work is needed to confirm the findings.

In summary, we have shown that cagA positive strains are associated with a dense infiltration of *H. pylori*, an enhanced inflammatory response, and gastroduodenal ulceration. The significance of the lack of association of bacterial density with peptic ulceration in this study is uncertain. The vacA s1 type is independently associated with increased inflammation but there was no correlation with density of bacterial colonisation.

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