

# Virulence markers of *Helicobacter pylori* in patients with diarrhoea-dominant irritable bowel syndrome

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## Introduction

*Helicobacter pylori* is a highly prevalent Gram-negative microaerophilic bacterium. It causes chronic gastric infection leading to gastritis, peptic ulcer and eventually gastric cancer.<sup>1-3</sup> *H. pylori* infection is associated with remodelling of gastric mucosal nerves<sup>4</sup> and stimulation of mast cells.<sup>5,6</sup> In animal models, *H. pylori* infection increases the neural responsiveness of smooth muscle.<sup>7</sup>

Virulence markers of *H. pylori* cytotoxin associated gene (*cagA*) and vacuolating cytotoxin gene (*vacA*) are associated with different gastroduodenal diseases.<sup>8,9</sup> Most *H. pylori* strains secrete a VacA cytotoxin that causes structural and functional alterations in epithelial cells and play an important role in the pathogenesis of *H. pylori*-associated gastroduodenal diseases.

Mutation of a hydrophobic region near the VacA amino terminus produces a mutant toxin that fails to induce cell vacuolation with defective functional activity.<sup>10</sup> VacA forms anion-selective channels in artificial planar lipid bilayers that increase the anion permeability of the HeLa cell plasma membrane and determines membrane depolarisation.<sup>11</sup>

When added to cultured cells, VacA induces vacuolation, an effect potentiated by pre-exposure of the toxin to low pH. The anion-selective, voltage-dependent pores formed in artificial membranes are potentiated by acidic conditions.<sup>12</sup> Pore formation is required both for cell vacuolation and increase of transepithelial conductivity.<sup>12</sup>

Purified pH-activated VacA, when added to Caco-2 cell monolayers, demonstrates enterotoxic effect.<sup>13</sup> This is time- and dose-dependent and is saturable.<sup>13</sup> Although VacA is produced by *H. pylori* in the stomach, it may exert an effect on more distal regions of the intestine. In gastric and small bowel epithelia, VacA is known to produce acidic ballooning of the endoplasmic reticulum and permeabilisation of the cell membrane, independent of the cytopathic effects.<sup>14</sup>

In children with diarrhoea, a cytotoxin-inducing vacuolation in HEp-2 cells was detected in 19 out of 618 stool specimens, and these resembled those induced by the *vacA* of *H. pylori*.<sup>15</sup>

The prevalence of *H. pylori* seropositivity in the authors'

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## ABSTRACT

Recent studies suggest that irritable bowel syndrome (IBS) is associated with low-grade inflammation. This study aims to determine the distribution of *Helicobacter pylori* cytotoxin-associated gene A (*cagA*) and vacuolating cytotoxin A (*vacA*) alleles (e.g., *s1* and *s2*) in patients with diarrhoea-dominant IBS (IBS-D) as the latter causes vacuolation in colonic epithelial cells *in vitro*. One hundred and seventy patients meeting Rome III criteria for IBS-D (mean age: 40 ± 15 years) were enrolled. Gastric biopsy was assessed histologically and DNA extraction was performed by polymerase chain reaction (PCR) for *H. pylori* genus 16S ribosomal DNA (16S rDNA), *cagA* and *vacA* allele *s1* and *s2*. There was no age- or gender-related difference in *H. pylori* positivity in IBS-D compared to the control group. *H. pylori* was positive in 116 (68%) with IBS-D compared to 88 (55%) in the control group ( $P=0.01$ ). *cagA* was positive in 73 (63%) with IBS-D compared to 42 (48%) in the control group ( $P=0.03$ ). *vacA s1* was positive in 61 (53%) with IBS-D compared to 32 (36%) in the control group ( $P=0.02$ ). *cagA s1* was positive in 39 (34%) with IBS-D compared to 13 (15%) in the control group ( $P=0.002$ ).

KEY WORDS: *cagA*.

*Helicobacter pylori*.

Irritable bowel syndrome.

*vacA* alleles.

local population in children aged 11–15 years is 53.5%.<sup>16</sup> In another study where *H. pylori* infection was established by rapid urease test and histopathology, *cagA*-negative *H. pylori* infection was present in the majority of non-ulcer dyspepsia patients; however, *cagA* was associated with peptic ulcer and gastric carcinoma.<sup>17</sup> *vacA* alleles *s1am1* and *s1bm1* are associated with *H. pylori*-associated disease and inflammation.<sup>17</sup>

The aim of the present study is to determine the distribution of the *H. pylori* virulence markers *cagA* and *vacA* in irritable bowel syndrome with diarrhoea (IBS-D) according to the Rome III criteria, compared to the distribution in patients with chronic diarrhoea.

## Materials and methods

A total of 330 patients were enrolled in the study, comprising 170 (52%) with IBS-D and 160 (48%) with chronic diarrhoea. Mean age of the IBS-D group was 41 ± 15 years (range: 16–83; males: 116, females: 54). Mean age of the 160 patients with chronic diarrhoea used as controls was 42 ± 14 years (range 15–75; males: 106, females: 54). Patients in the control group had abdominal pain or discomfort associated with

intermittent diarrhoea that did not fulfill the Rome III criteria for IBS-D.

Patients meeting the Rome III criteria for IBS-D had symptoms for at least three months, with onset at least six months previously.<sup>18</sup> These patients underwent thorough history, physical examination, complete blood count, serum creatinine, electrolytes, stool microscopy, oesophago-gastroduodenoscopy (EGD) and colonoscopy. On EGD, gastric and duodenal biopsies were taken for *H. pylori* infection, coeliac disease and giardiasis. On colonoscopy, rectal biopsy was taken for the diagnosis of inflammatory bowel disease, microscopic colitis and infective colitis. They also had serology for tissue transglutaminase (TTG) IgA and IgG antibodies for coeliac disease.

Gastric biopsy specimens were used for histopathology for the diagnosis of *H. pylori* and DNA extraction for the polymerase chain reaction (PCR) to amplify *H. pylori* genes for ribosomal DNA (rDNA), *cagA* and *vacA* alleles *s1* and *s2*.

Gastritis was graded on a four-point scale of none (grade 0), mild (grade 1), moderate (grade 2), and severe (grade 3) according to the Sydney guidelines.<sup>19</sup> The presence of *H. pylori* was assessed on haematoxylin and eosin (H&E)-stained sections. Diagnosis of *H. pylori* infection was established when PCR for 16S rDNA or a specific PCR for *H. pylori* was positive.

Exclusion criteria included previous history of peptic ulcer disease, history of treatment for *H. pylori*, concurrent or recent antibiotic use (e.g., metronidazole, clarithromycin, amoxicillin, tetracycline, doxycycline and other cephalosporins), histamine-2 receptor blocker or proton pump inhibitor therapy, and concurrent infection with *Blastocystis hominis* or *Giardia lamblia*.

The study was approved by the institutional ethics review committee.

#### Faecal smear microscopy

Briefly, approximately 2 mg faeces was emulsified on a glass slide in one drop of physiological saline and covered with a cover slip. A similar preparation was made on another slide using Lugol's iodine. These preparations were examined

under low power (x10) and high power (x40) objective lenses.

#### Extraction of genomic DNA

The extraction of DNA was performed as described previously.<sup>20</sup> Briefly, gastric tissue was homogenised in 500 µL sterile water and centrifuged at 12,000 xg for 3 min. Then, 500 µL lysis buffer (100 mmol/L NaCl, 10 mmol/L Tris-HCl [pH 8.0], 25 mmol/L EDTA, 0.5% sodium dodecyl sulphate) and 10 µL proteinase K (10 mg/mL) were added. Incubation was carried out at 50°C for 20 h, followed by phenol-chloroform extraction and ethanol precipitation. The resulting pellet was allowed to dissolve in 40 µL TE buffer (10 mmol/L Tris-HCl [pH 7.4], 0.1 mmol/L EDTA [pH 8.0]) for 20 h at 37°C. Samples were stored at -20°C prior to PCR amplification. The DNA content and purity were determined by measuring the absorbance at 260 nm and 280 nm, respectively, using a spectrophotometer (Beckman DU-600, USA).

#### PCR amplification

The PCR reaction was performed using extracted DNA as the template. Samples that were positive for *Helicobacter* genus 16S rDNA were subsequently analysed with different primers to detect the *cagA*, *cagA* empty site and *vacA* alleles (e.g., *s1* and *s2*) (Table 1) which encode potential virulence factors in *H. pylori*.<sup>21,22</sup> If *cagA* was negative, PCR was performed to confirm the presence of *cagA* empty site in *cagA*-negative strains, and to eliminate cases of amplification failure. Primers used were those described previously.<sup>23,24</sup>

Amplification was carried out in a total volume of 50 µL containing 2 mM dNTPs, 1 mM 50 pmol each forward and reverse primer<sup>25,26</sup> (synthesised by MWG automatic synthesiser), 1 unit *Thermus aquaticus* (*Taq*) DNA polymerase (Promega), 5 µL 10x PCR reaction buffer, 3 mM MgCl<sub>2</sub> and 2 µL DNA template containing 0.5 ng extracted DNA. Total volume was increased to 50 µL by the addition of double-distilled water. The reaction was carried out in a Perkin Elmer 9700 thermal cycler, and the amplification cycles for the various *H. pylori* genes are shown in Table 1.

**Table 1.** Oligonucleotide primers used in typing *H. pylori* genes.

Region amplified	Primer designation	Primer sequences (5'→3')	PCR product (bp)	PCR cycles
16S rRNA <sup>21</sup>	C97	GCT ATG ACG GGT ATC C	400	94°C for 5 min, 94°C for 1 min, 55°C for 1 min, 72°C for 90 sec (35 cycles), 72°C for 7 min
	C 98	GAT TTT ACC CCT ACA CCA		
<i>vacA</i> alleles <sup>22</sup>	S1	ATGGAAATACAACAAACACAC	259 <sup>19</sup>	One cycle of 95°C for 5 min, 35 cycles of 95°C for 1 min, 52°C for 1 min, 72°C for 1 min, and one cycle of 72°C for 5 min
	S2	CTGCTTGAATGCGCCAAAC	286 <sup>19</sup>	
<i>cagA</i> <sup>23</sup>	D008	GGTCAAAATGCGGTTCATGG	297 <sup>19</sup>	One cycle of 94°C for 5 min, 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, and one cycle of 72°C for 5 min
	R008	TTAGAATAATCAACAAACATCACGCCAT		
Empty site <sup>24</sup>	ES-F	ACATTTTGGCTAAATAAACGCTG	360	One cycle of 94°C for 5 min, 40 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, and one cycle of 72°C for 5 min
	ES-R	TCATGCGAGCGCGATGTG		

Positive and negative control reactions were performed with each batch of amplifications. DNA from *H. pylori* strains ATCC 43504 (*vacAs1am1*, *cagA*-positive), ATCC 51932 (*vacA s2m2*, *cagA*-negative) and ATCC 43526 (*vacA s1bm1*, *cagA*-positive) was used to define the accuracy of the *cagA* and *vacA* alleles. After PCR, the amplified products were electrophoresed in 2% agarose gels containing 0.5% Tris/acetate/ethylenediaminetetraacetic acid, stained with ethidium bromide, and visualised under an ultraviolet (UV) light source.

#### Sample size

Using NCSS (PASS) software for the sample size calculation, a group of 330 subjects was required to achieve 80% power to detect a difference of 15% that prevalence of *H. pylori* in IBS patient would be 69% when compared to the non-IBS group<sup>16,25</sup> at a 5% level of significance.

#### Statistical analysis

Results were expressed as mean+standard deviation (SD) for continuous variables (e.g., age) and number (percentage) for categorical data (e.g., gender, stool culture, diarrhoea). Univariate analysis was performed using the independent sample *t*-test, Pearson  $\chi^2$  test and Fisher Exact test where appropriate.  $P < 0.05$  was considered statistically significant. All *P* values were two sided. Statistical interpretation of data was performed using SPSS version 16.0.

## Results

On EGD, *H. pylori* gastritis was present in 162 (49%) patients and non-specific gastritis was present in 168 (51%). At colonoscopy, 138 (42%) had non-specific inflammation while 192 (58%) were reported as normal (Table 2). On histopathology, gastric biopsy revealed chronic active gastritis in 90 (53%) and chronic gastritis in 80 (47%) patients with IBS-D compared to 63 (39%) and 97 (61%), respectively, in patients with chronic diarrhoea ( $P=0.02$ ). Colonic biopsies revealed non-specific inflammation in 90 (53%) and normal colonic mucosa in 80 (47%) in patients with IBS-D compared to 102 (64%) and 58 (36%), respectively, in patients with chronic diarrhoea ( $P=0.05$ ) (Table 2).

#### Prevalence of infection

*H. pylori* was positive in 116 (68%) with IBS-D compared to 88 (55%) in the control group ( $P=0.01$ ). It was associated with *H. pylori*-positive gastritis in 91 (54%) patients with IBS-D compared to 71 (44%) in the control group ( $P=0.09$ ) (Table 2).

#### Distribution of virulence markers

*cagA* was positive in 115 (56%), *cagA* empty site in 89 (44%), *vacAs1* in 93 (46%), *vacAs2* in 124 (61%), *cagAs1* in 53 (26%) and *cagAs2* in 68 (33%) (Figs. 2 and 3). *cagA* was positive in 73 (63%) patients with IBS-D compared to 42 (48%) in the control group ( $P=0.03$ ) (Table 2). *H. pylori* infection with *cagA* empty site was positive in 43 (37%) in patients with IBS-D and 46 (52%) in the control group ( $P=0.03$ ). *vacAs1* was positive in 61 (53%) patients with IBS-D compared to 32 (36%) in the control group ( $P=0.02$ ) (Table 2). *cagAs1* was positive in 39 (34%) patients with IBS-D compared to 13 (15%) in the control group ( $P=0.002$ ) (Table 2).

**Table 2.** Details of patients enrolled in the study.

	IBS (n=170)	Controls (n=160)	<i>P</i> value
<b>Age</b>			
Mean±SD	40±15	42±14	
Range	16–83	15–75	
<b>Gender</b>			
Male	116(69)	106(66)	
Female	54(31)	54(34)	
<b>EGD</b>			
<i>H. pylori</i> -positive gastritis	91(54)	71(44)	0.09
<i>H. pylori</i> -negative gastritis	79(46)	89(56)	
<b>Colonoscopy</b>			
Normal	80(47)	58(36)	0.05
Non-specific	90(53)	102(64)	
<b>PCR for <i>Helicobacter pylori</i></b>			
<b>16S rDNA</b>			
Positive	116(68)	88(55)	0.01
Negative	54(43)	72(45)	
<b><i>cagA</i></b>			
Positive	73(63)	42(48)	0.03
Negative	43(37)	46(52)	
<b><i>cagA</i>-empty site</b>			
Positive	43(37)	46(52)	0.03
Negative	73(63)	42(48)	
<b><i>vacAs1</i></b>			
Positive	61(53)	32(36)	0.02
Negative	55(47)	56(64)	
<b><i>vacAs2</i></b>			
Positive	67(58)	57(65)	0.31
Negative	49(42)	31(35)	
<b><i>cagAs1</i></b>			
Positive	39(34)	13(15)	0.002
Negative	77(66)	75(85)	
<b><i>cagAs2</i></b>			
Positive	38(33)	30(34)	0.84
Negative	78(67)	58(66)	
Univariate analysis was performed using the independent sample <i>t</i> -test; Pearson $\chi^2$ test and Fisher Exact test were also used where appropriate. $P < 0.05$ was considered statistically significant. Percentage shown in parentheses.			

#### Comparison of infection in different groups

No age- or gender-related differences in *H. pylori* positivity were seen, nor differences in *H. pylori*-associated gastritis in the two groups (Tables 2 and 3). *cagA* was positive in 73 (63%) patients with IBS-D compared to 42 (48%) in the control group ( $P=0.03$ ), while *vacAs1* was positive in 61 (53%) with IBS-D compared to 32 (36%) in the control group ( $P=0.02$ ) (Table 3). *cagAs1* was positive in 39 (34%) patients with IBS-D compared to 14(16%) in the control group ( $P=0.004$ ) (Table 3).

## Discussion

The implications of the study reported here are that *H. pylori* infection is associated with *H. pylori* strains that are *cagAs1*-positive, compared to a previously reported increased incidence of *cagA*-negative *H. pylori* strains in patients with non-ulcer dyspepsia.<sup>17</sup> It shows variation in the distribution of *H. pylori* virulence markers in IBS-D compared to non-ulcer dyspepsia.

There is evidence to suggest that *H. pylori* vacuolating toxin has a role in causing diarrhoea as it produces vacuolation in colonic epithelial cells. However, the present study did not demonstrate this effect, and was only able to show an increase in distribution of *H. pylori* virulence markers in IBS-D patients.

It is known that that co-infection with parasitic organisms tends to modulate the immune response;<sup>27</sup> however, patients in the present study did not show evidence of infestation (e.g., *Blastocystis hominis*) on stool examination.

Irritable bowel syndrome is a heterogeneous disorder and not all patients with IBS give a history of gastroenteritis, pelvic surgery and psychological trauma.<sup>26</sup> It is possible that *H. pylori* infection serves to sensitise the bowel and leads to the appearance of IBS symptoms.<sup>28</sup> The VacA cytotoxin secreted by *H. pylori* strains may contribute to diarrhoea in IBS-D following structural and functional alterations to intestinal epithelial cells that increase their permeability.<sup>11</sup>

In conclusion, the present study showed that *H. pylori* infection is common in patients with IBS-D, and is associated with virulent strains that are predominantly *cagAs1*-positive. However, further studies are required to illustrate the effect of *H. pylori* virulence markers in patients with IBS. □

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**Table 3.** Comparison of *Helicobacter* positivity.

	IBS (n=116)	Controls (n=88)	P value
<b>Age (years)</b>			
≤42	68(59)	50(57)	0.80
≥43	48(41)	38(43)	
<b>Gender</b>			
Male	79(68)	50(63)	0.40
Female	37(32)	33(37)	
<b>EGD</b>			
<i>H. pylori</i> -positive gastritis	27(23)	18(21)	0.63
<i>H. pylori</i> -negative gastritis	89(77)	70(79)	
<b>Colonoscopy</b>			
Normal	51(44)	28(32)	0.08
Non-specific	65(56)	60(68)	
<b>PCR for <i>Helicobacter pylori</i></b>			
<b>16S rDNA</b>			
Positive	116(68)	88(55)	0.01
Negative	54(43)	72(45)	
<b><i>cagA</i></b>			
Positive	73(63)	42(48)	0.03
Negative	43(37)	46(52)	
<b><i>cagA</i>-empty site</b>			
Positive	43(37)	46(52)	0.03
Negative	76(63)	42(48)	
<b><i>vacAs1</i></b>			
Positive	61(53)	32(36)	0.02
Negative	55(47)	56(64)	
<b><i>vacAs2</i></b>			
Positive	67(58)	57(65)	0.31
Negative	49(42)	31(35)	
<b><i>cagAs1</i></b>			
Positive	39(34)	14(16)	0.004
Negative	77(66)	74(84)	
<b><i>cagAs2</i></b>			
Positive	38(33)	30(34)	0.84
Negative	78(67)	58(66)	
Univariate analysis was performed using the independent sample <i>t</i> -test; Pearson $\chi^2$ test and Fisher Exact test were also used where appropriate. <i>P</i> <0.05 was considered statistically significant. Percentage shown in parentheses.			

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