

## ORIGINAL ARTICLE

# Topographical localisation of *cagA* positive and *cagA* negative *Helicobacter pylori* strains in the gastric mucosa; an in situ hybridisation study

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**Background:** The *cagA* gene is a marker for the presence of the *cag* pathogenicity island, and the presence of *cagA* positive strains of *Helicobacter pylori* can identify individuals with a higher risk of developing gastrointestinal diseases.

**Aims:** To study the interaction between *H pylori* *cagA*(+) and *cagA*(-) strains and the gastric mucosa.

**Methods:** Patients with *H pylori* associated gastritis and peptic ulcers were studied. Biopsies were obtained from the antrum, corpus, fundus, and incisura for *H pylori* culture, and for in situ hybridisation studies. From each biopsy, multiple single *H pylori* colonies were isolated and propagated for DNA isolation, and *cagA* was detected by the polymerase chain reaction (PCR). For in situ detection of *H pylori* an oligonucleotide specific for an *H pylori* common antigen and an oligonucleotide specific for *cagA* were used as probes. Biotinylated probes were incubated with biopsy sections, developed with streptavidin-horseradish peroxidase, and amplified with the tyramide system.

**Results:** PCR results for *cagA* in isolated colonies confirmed the in situ hybridisation studies. In situ hybridisation identified *cagA*(+) bacteria in patients with *cagA*(+) isolates; *cagA*(-) bacteria in patients with *cagA*(-) isolates, and *cagA*(+) and *cagA*(-) bacteria in patients with both *cagA*(+) and *cagA*(-) isolates. *CagA*(-) bacteria usually colonised the mucous gel or the apical epithelial surface, whereas *cagA*(+) bacteria colonised the immediate vicinity of epithelial cells or the intercellular spaces.

**Conclusions:** These results document a different in vivo interaction between *H pylori* *cagA*(+) or *cagA*(-) strains and the gastric mucosa.

The organism *Helicobacter pylori* colonises the stomach mucosa of over 50% of the world's population; peptic ulcers develop in about 10–15% of these infected individuals and gastric carcinoma in less than 3% of patients.<sup>1</sup> Differences exist in the genotypes of *H pylori* strains, and the presence of *vacA* and of the *cag* pathogenicity island (*cag* PAI) is linked to the development of peptic ulcers and gastric carcinoma.<sup>2–4</sup> Infection with *cag* PAI (+) strains is associated with increase interleukin 8 secretion by epithelial cells, which recruit inflammatory cells,<sup>5</sup> and released interleukins may lead to cell damage, apoptosis, and DNA damage.

"The aim of our study was to develop an in situ hybridisation assay to differentiate between colonisation with *Helicobacter pylori* *cagA*(+) and *cagA*(-) strains in gastric biopsies"

The *cagA* gene is a marker for the presence of the *cag* PAI, and the detection of *cagA* positive strains may help to identify individuals with a higher risk of developing gastrointestinal diseases. Until now, the identification of infection with *cagA*(+) strains has been done by the isolation and culture of strains from biopsies and the detection of the gene by polymerase chain reaction (PCR) or hybridisation techniques.<sup>6–7</sup> However, the in situ localisation of *cagA*(+) and (–) strains in the gastric mucosa has not been studied.

In situ hybridisation techniques using probes for specific genes allow the in situ identification of virulent versus non-virulent infectious organisms and provide additional morphological information. The aim of our study was to develop an in situ hybridisation assay to differentiate between colonisation with *H pylori* *cagA*(+) and *cagA*(–) strains in gastric biopsies.

## MATERIALS AND METHODS

### Gastric biopsy specimens

Gastric biopsies were obtained from patients attending the Hospital de Especialidades, IMSS in Mexico City. Patients infected with *H pylori*, with non-ulcer dyspepsia, duodenal ulcer, or gastric ulcer were studied. From each patient, two biopsy specimens were obtained from the antrum, corpus, fundus, and incisura. From each region, one biopsy specimen was used for bacterial culture of *H pylori*, whereas the second was fixed and embedded in paraffin wax and used for in situ hybridisation studies. As negative controls, biopsies from two patients with *H pylori* negative gastritis were studied.

### Isolation of multiple single colonies of *H pylori* from biopsies

Biopsies were inoculated on to blood agar plates with antibiotics. From the primary growth, seven to 10 single colonies were propagated on blood agar medium. The identity of the strains was confirmed by Gram stain, urease, oxidase, and catalase tests. Colonies were swept and suspended in saline solution for DNA isolation.

### PCR for *cagA*

Primers used for PCR typing of *cagA* and for the empty site to document the absence of *cag* PAI were described previously.<sup>8,9</sup> PCR conditions for the *cagA* primers were 35 cycles of 94°C for 30 seconds, 55°C for one minute, and 72°C for 1.5 minutes, followed by a final extension at 72°C for six minutes. For the *cag* PAI empty site, the PCR conditions were 35 cycles of 94°C for one minute, 57°C for one minute, and

**Abbreviations:** FITC, fluorescein isothiocyanate; HRP, horseradish peroxidase; PAI, pathogenicity island; PCR, polymerase chain reaction

**Table 1** Correlation between genotyping for cagA by PCR in isolated single colonies and by in situ hybridisation in biopsy sections from the stomach

Patient	Disease	Site	PCR cagA*	In situ hybridisation	
				cagA(+)	cagA(-)
249	DU	Antrum	+	+	—
		Corpus	+	ND	ND
		Fundus	+	+	—
		Incisura	+	+	—
251	DU	Antrum	—	—	+
		Corpus	—	—	+
		Fundus	—	—	+
		Incisura	—	—	+
252	GU	Antrum	—	+	+
		Corpus	—	—	+
		Fundus	—	—	+
		Incisura	—	—	+
254	NUD	Antrum	—	—	+
		Corpus	—	—	+
		Fundus	—	—	+
		Incisura	—	—	+
256	DU	Antrum	+	+	—
		Corpus	+	+	—
		Fundus	+	+	—
		Incisura	+	+	—
259	DU	Antrum	+	+	+
		Corpus	+/-	+	+
		Fundus	+/-	+	+
		Incisura	+/-	+	+
261	GU	Antrum	+	+	—
		Corpus	+	+	—
		Fundus	+	+	—
		Incisura	+	ND	ND

\*Results of PCR test for cagA to single colonies isolates; 7 to 10 colonies were isolated from each biopsy site. DU, duodenal ulcer; GU, gastric ulcer; ND, not determined; NUD, non-ulcer dyspepsia; PCR, polymerase chain reaction.

72°C for one minute, followed by a final extension at 72°C for six minutes. Amplified products were analysed in 2% agarose gels.

### DNA probes for in situ hybridisation

For the detection of all *H. pylori* strains a 303 bp oligonucleotide specific for an antigen common to all *H. pylori* strains<sup>10</sup> was used as a probe (Ag-C probe). For the detection of cagA(+) strains a 349 bp oligonucleotide specific for cagA<sup>9</sup> was used as a probe. The probes were labelled with biotin and were synthesised by Maxim Biotec Inc (San Francisco, California, USA).

### Bacteria

*Helicobacter pylori* 60190 (ATCC 49503) genotype cagA(+) and *H. pylori* Tx30a (ATCC 51932) genotype cagA(-) were used for evaluation of the probe specificity. Strains were cultured and suspended in saline solution; mixtures of different proportions of each strain were prepared and spotted on to glass slides and immediately fixed in acetone.

### In situ hybridisation using bacteria fixed on glass slides

Slides were incubated with proteinase K (20 µg/ml) for one minute, washed, and the cagA probe (4 µg/µl) was added; for DNA denaturation, slides were incubated for five minutes at 95°C, and at 4°C for 10 minutes. Hybridisation was carried out at 37°C for two hours in a humid chamber. Stringent washing was carried out and the biotinylated probes were detected with streptavidin-horseradish peroxidase (HRP)

(NEN Inc, Boston, Massachusetts, USA), followed by Cy3-tyramide amplification reagent<sup>11</sup> (NEN Inc) for 15 minutes at 37°C. Slides were washed and the Ag-C probe (4 µg/µl) was added. Denaturation, hybridisation, and stringent washing were done as described for the cagA probe. The second probe was detected with streptavidin-HRP, followed by the fluorescein isothiocyanate (FITC)-tyramide amplification reagent for 15 minutes at 37°C. Slides were analysed with an epifluorescence or confocal microscope (Carl Zeiss, Jena, Germany).

### In situ hybridisation in gastric biopsies

Sections (4 µm thick) of each biopsy were mounted on charged slides. Sections were dewaxed, dehydrated, and washed. Slides were placed in Declere (Cell Marque Corp, Austin, Texas, USA) for 15 minutes, washed, and treated with proteinase K (25 µg/ml) for one minute. Endogenous peroxidase was blocked using 3% H<sub>2</sub>O<sub>2</sub>. To block biotin, sections were incubated with avidin for 15 minutes, whereas to block avidin they were incubated with biotin for 15 minutes. Sections were covered with 10 µl of the cagA probe (4 µg/µl). For DNA denaturation, sections were incubated at 95°C for five minutes, and at 4°C for 10 minutes. Slides were then hybridised overnight at 37°C in a humid chamber and washed with HWb (Research Genetics Inc, Huntsville, Alabama, USA) at 60°C for 10 minutes. Sections were incubated in protein blocking buffer (Bio SB Inc, Santa Barbara, California, USA) for 30 minutes, and incubated with streptavidin-HRP for 30 minutes. After washing, slides were incubated with Cy3-tyramide for 15 minutes at 37°C and



**Figure 1** In situ hybridisation for the detection of cagA(+) and cagA(-) strains in a mixture of *Helicobacter pylori* ATCC 49503 (cagA(+)) and Tx30a (cagA(-)) strains fixed on glass slides. The DNA probe for the common antigen was developed with fluorescein isothiocyanate (green) and the probe for cagA was developed with Cy3 (red). (A) green filter; (B) red filter; and (C) triple filter, yellow areas indicate overlapping of the green common antigen and the red cagA, thereby demonstrating the presence of cagA(+) strains.

washed. Slides were incubated with 0.1% saponin for five minutes and washed. The second biotinylated probe for *H. pylori* detection (Ag-C probe) was applied (4 µg/µl); hybridisation steps were the same as those for the cagA probe. For detection, FITC-tyramide was applied and developed using similar conditions as for Cy3-tyramide. After washing, slides were counterstained with DAPI and covered with vectashield. The slides were observed with an epifluorescence and a confocal microscope (Carl Zeiss).

## RESULTS

### PCR for cagA in single colonies

In total, 224 *H. pylori* strains were isolated from the four regions of the stomach of the seven patients studied (table 1). All strains tested from three patients (249, 256, and 261) were positive for cagA by PCR. All strains from three patients (251, 252, and 254) were negative for cagA. In patient 259, strains isolated from the antrum were all cagA(+), whereas strains isolated from the corpus, fundus, and incisura were a mixture of both cagA(+) and cagA(-) strains (table 1). In all cagA(-) strains the absence of the cag PAI was confirmed by amplification of the "empty site" with specific primers.

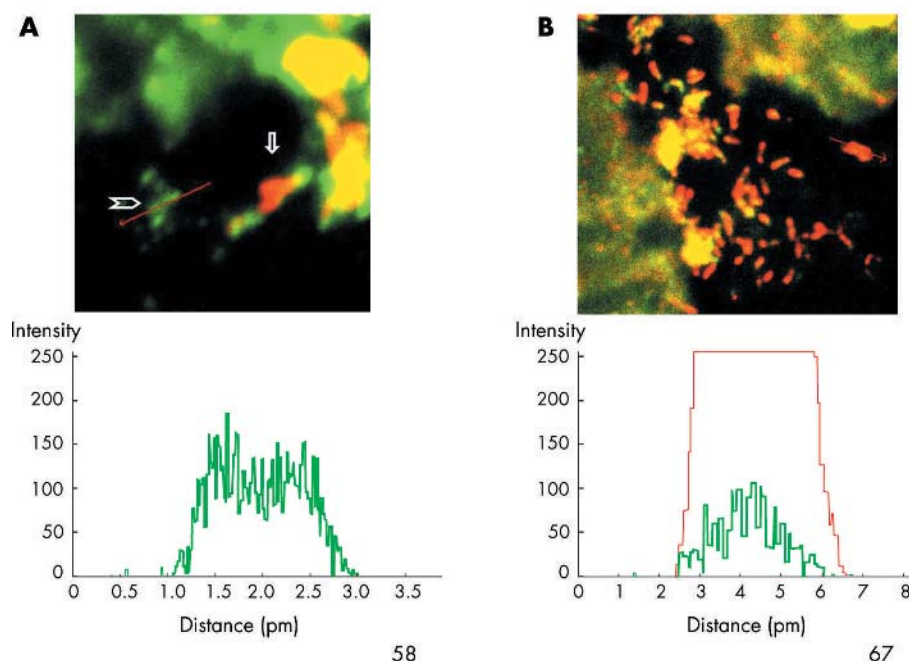
### In situ hybridisation in bacteria fixed on slides

The performance of the probes specific for the common antigen and for cagA was tested with *H. pylori* 60190 strain (cagA(+)) and strain Tx30a (cagA(-)). Suspensions with different proportions of each strain were fixed and tested. The probes were able to differentiate between cagA(-) and cagA(+) strains (fig 1).

### In situ hybridisation in gastric biopsies

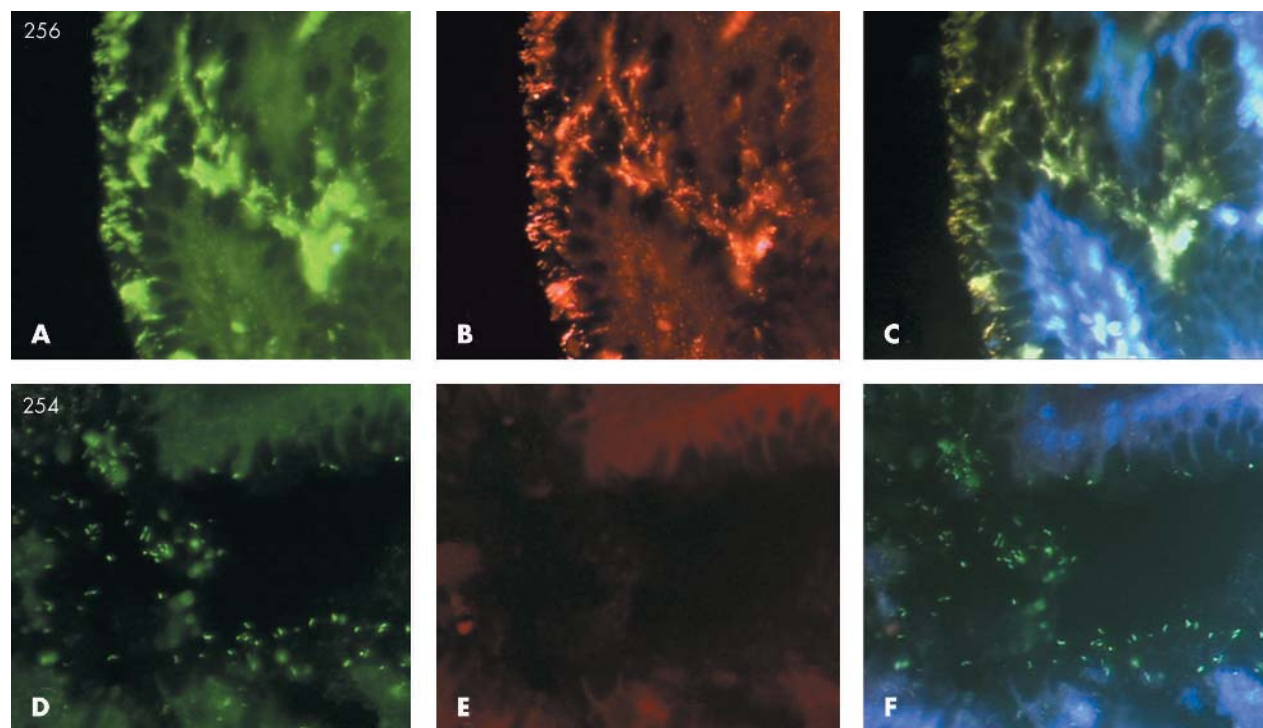
No *H. pylori* bacteria were seen in the biopsies of the two patients without *H. pylori* infection. To document the specificity of the staining, bacteria in biopsies from patients colonised with either the cagA(+) or cagA(-) strains were scanned and the intensity of the FITC and Cy3 fluorescence was measured (fig 2). Patient 58 was colonised with both cagA(-) and cagA(+) strains and the scanning of a bacterium revealed green with the triple filter, confirming the exclusive presence of FITC fluorescence (fig 2A); patient 67 was colonised with cagA(+) strains and the scanning of bacteria with the triple filter demonstrated the presence of both FITC and Cy3 fluorescence (fig 2B).

Table 1 presents the correlation between the cagA PCR results in the single isolates and the in situ hybridisation tests



**Figure 2** In situ hybridisation for the detection of *Helicobacter pylori* cagA(+) and cagA(-) strains in gastric biopsies of patients colonised with *H. pylori*. The DNA probe for the common antigen was developed with fluorescein isothiocyanate (FITC; green) and the probe for cagA was developed with Cy3 (red). (A) The gastric biopsy of a patient colonised with both cagA(+) and cagA(-) strains; a cagA(-) bacterium was scanned to show the presence of FITC and the absence of Cy3 fluorescence; the arrowhead points to cagA(-) and the arrow to cagA(+) bacteria. (B) The gastric biopsy of a patient colonised exclusively with cagA(+) strains; a bacterium was scanned to show the presence of both FITC and Cy3 fluorescence.



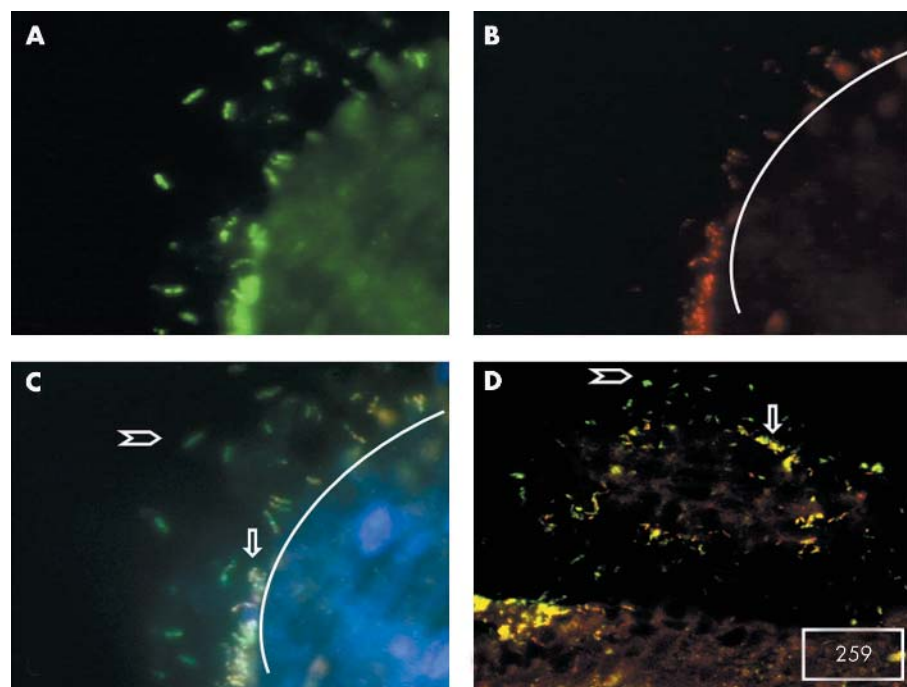


**Figure 3** In situ hybridisation for the detection of *Helicobacter pylori* cagA(+) and cagA(-) strains in gastric biopsies of patients colonised with *H. pylori*. The DNA probe for the common antigen was developed with fluorescein isothiocyanate (FITC; green) and the probe for cagA was developed with Cy3 (red). (A, D) Green filter; (B, E) red filter; and (C, F) triple filter, the yellow areas indicate overlapping of the green common antigen and the red cagA, thereby demonstrating the presence of cagA(+) strains. Patient 256 was colonised with cagA(+) strains, and colonisation is seen close to the epithelium; patient 254 was colonised with cagA(-) strains, and colonisation is seen mainly in the mucous.

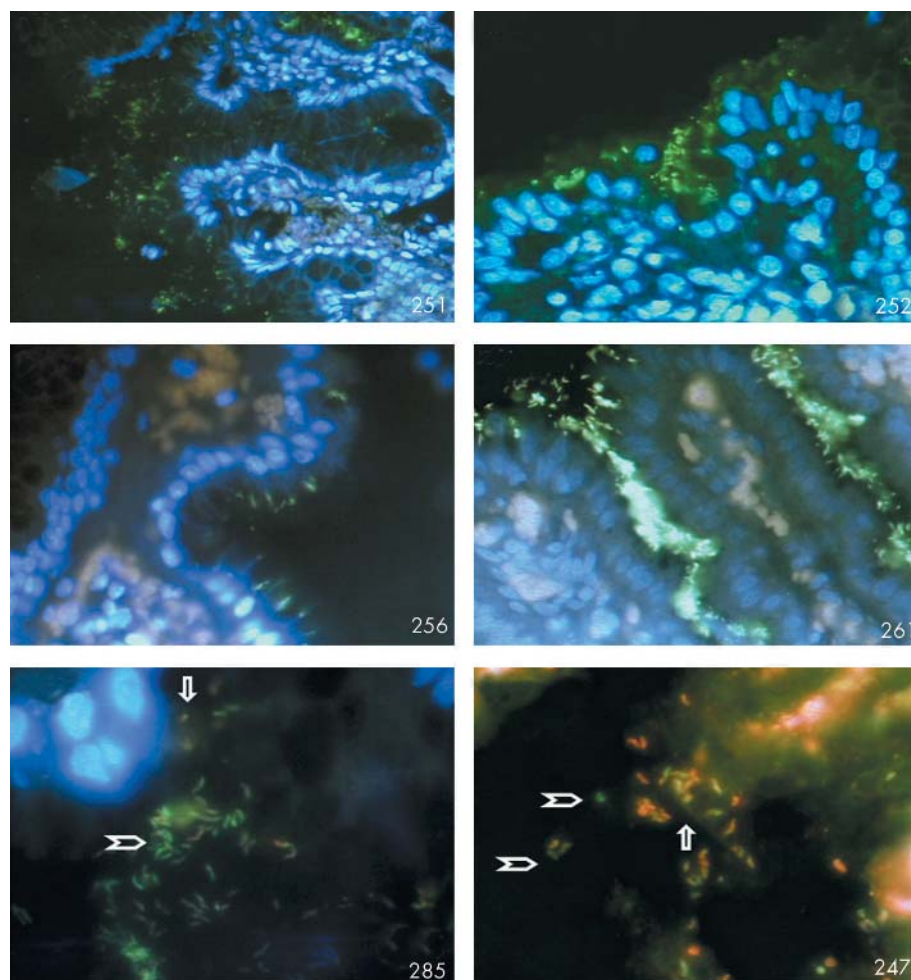
for seven patients studied in four stomach regions. PCR results were confirmed by the in situ hybridisation studies with two exceptions. In patient 252, all single isolates were cagA(-) by PCR but some cagA(+) bacteria were identified in the antrum by in situ hybridisation. In patient 259, all single isolates from the antrum were cagA(+) by PCR but in situ

hybridisation demonstrated the presence of some cagA(-) bacteria.

Figure 3 shows the results of biopsies from two patients observed with FITC, Cy3, and the triple filter. In patient 256, all the isolates were cagA(+) by PCR, and the bacteria were seen in situ with all three filters (fig 3A-C), as expected;



**Figure 4** In situ hybridisation for the detection of *Helicobacter pylori* cagA(+) and cagA(-) strains in the gastric biopsy of a patient (259) colonised with both cagA(+) and cagA(-) strains. The DNA probe for the common antigen was developed with fluorescein isothiocyanate (FITC; green) and the probe for cagA was developed with Cy3 (red). (A) green filter; (B) red filter; (C) triple filter, yellow areas indicate overlapping of the green common antigen and red cagA, thereby demonstrating the presence of cagA(+) strains; and (D) triple filter in confocal microscopy. The arrowhead points to cagA(-) and the arrow to cagA(+) bacteria. cagA(-) strains are seen colonising mainly the mucous, whereas cagA(+) strains are in intimate contact with epithelial cells.



**Figure 5** In situ hybridisation for the detection of *Helicobacter pylori* cagA(+) and cagA(-) strains in gastric biopsies of patients colonised with *H. pylori*. The DNA probe for the common antigen was developed with fluorescein isothiocyanate (FITC; green) and the probe for cagA was developed with Cy3 (red). All preparations were observed with the triple filter. Patients 251 and 252 were colonised with cagA(-) strains, and colonisation is seen mainly in the mucous or in the proximity of the epithelial cells. Patients 256 and 261 were colonised with cagA(+) strains, and colonisation is seen in the interepithelial spaces and in intimate contact with the epithelium. Patients 285 and 247 were colonised with both, cagA(-) and cagA(+) strains. The arrowheads point to cagA(-) and the arrows to cagA(+) bacteria; in patient 285 some cagA(+) bacteria are seen in intimate contact with the epithelial cells; whereas in patient 247 a few cagA(-) bacteria are seen in the mucous.

colonisation was seen in the immediate vicinity of the epithelial cells. In patient 254, where all isolates were cagA(-) by PCR, bacteria were seen in situ with FITC and the triple filter but not with the Cy3 filter (fig 3D-F), and colonisation was seen mainly in the mucous gel.

Figure 4 shows the results of biopsies from patient 259 in whom isolates were both cagA(+) and cagA(-). All bacteria were stained when the FITC filter was used (fig 4A), whereas with Cy3 filter (fig 4B) only those bacteria in the immediate vicinity of the epithelial cells were stained (cagA(+) bacteria); these results were confirmed when the triple filter was used (fig 4C). Confocal microscopy (fig 4D) further documented the differential distribution of cagA(+) and cagA(-) bacteria.

Figure 5 shows further examples of the interaction of cagA(+) and cagA(-) bacteria with the gastric mucosa. In patients 251 and 252, all isolates were shown to be cagA(-) by PCR (table 1); in patient 251, the bacteria colonise mainly the mucous gel, whereas in patient 252 the bacteria are seen in the mucous and near the apical surface of the epithelial cells. In patients 256 and 261 all isolates were cagA(+) by PCR (table 1) and in both cases colonisation is seen in the immediate vicinity of the epithelium, in addition to the intercellular epithelial spaces. Two additional cases (285 and 247) with a mixed infection (cagA(+) and cagA(-)) are presented. In patient 285 colonisation with cagA(-) bacteria in the mucous gel predominates, although some cagA(+) bacteria can be seen in close contact with the epithelial cells; in patient 247, cagA(+) bacteria predominate and the few cagA(-) bacteria are seen in the mucous gel. Figure 6 shows

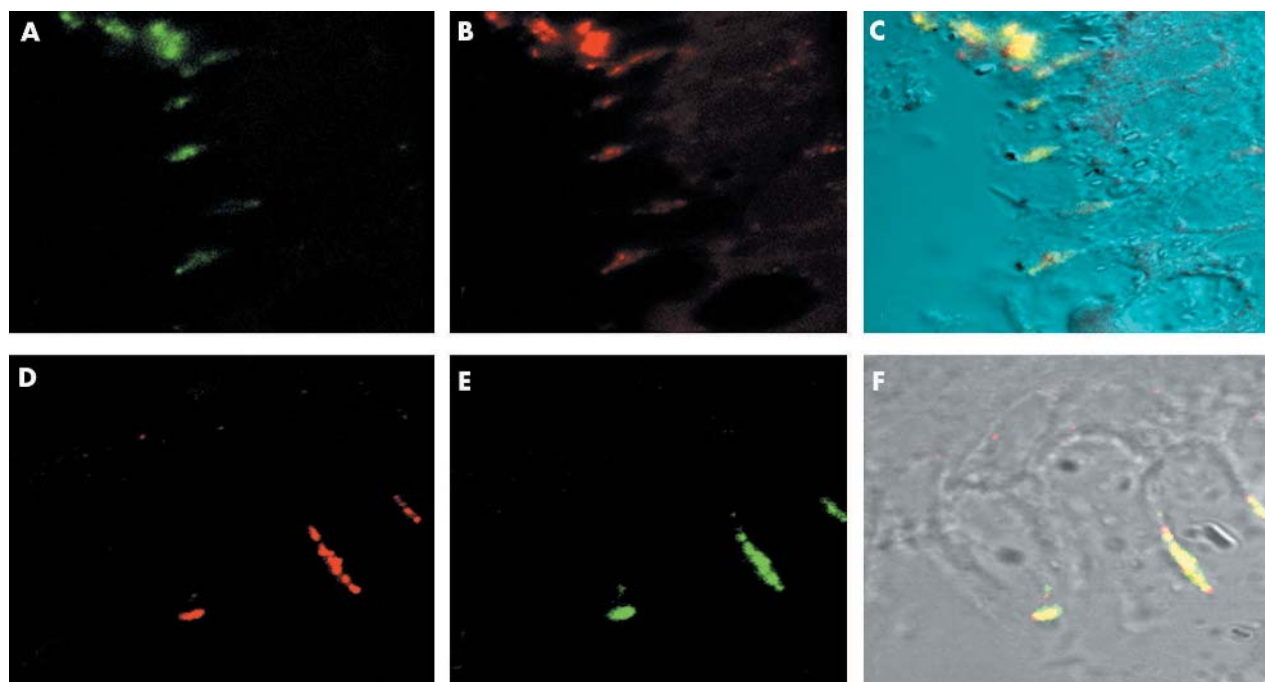
the pattern of colonisation of some of the cagA(+) bacteria in patient 285, and colonisation of intercellular epithelial spaces is seen.

## DISCUSSION

The presence of the cagA gene is a useful marker for the cag PAI, and gastric colonisation with cagA(+) strains has been documented by PCR in either isolated bacteria or DNA extracted from biopsies.<sup>6,7</sup> However, this approach does not provide information on the in vivo localisation of either cagA(+) or cagA(-) *H. pylori* strains. The possibility of in situ localisation of cagA(+) or (-) strains is important in the light of evidence that patients may be colonised with both cagA(+) and (-) strains.<sup>12,13</sup> In our study, we report a technique that allows the in situ detection and differentiation of both cagA(+) and (-) strains in gastric biopsies of patients infected with *H. pylori*. Results of in situ hybridisation were confirmed with PCR in multiple isolates from biopsies, with two exceptions, namely: (1) case 252, where PCR showed that all the isolates from the antrum were cagA(-), whereas in situ hybridisation documented colonisation with both cagA(+) and (-) strains; and (2) case 259, where all the isolates from the antrum were cagA(+), whereas in situ hybridisation showed the presence of both cagA(+) and (-) strains. These results suggest that the sensitivity of culture, even after testing multiple isolates, to identify strains with different genotypes is lower than that of in situ hybridisation.

In our study, initial attempts at in situ hybridisation of both the cagA(+) and (-) strains using probes directly





**Figure 6** In situ hybridisation for the detection of *Helicobacter pylori* cagA(+) and cagA(-) strains in gastric biopsies of patient 285. The DNA probe for the common antigen was developed with fluorescein isothiocyanate (FITC; green) and the probe for cagA was developed with Cy3 (red). (A, D) green filter; (B, E) red filter; (C, F) triple filter, the yellow areas indicate the overlapping of the green common antigen and the red cagA, thereby demonstrating the presence of cagA(+) strains. Two different regions of the epithelium are presented to show colonisation in the intercellular epithelial space.

labelled with fluorochromes gave unsatisfactory results. The technique was greatly improved when we used the tyramide method to amplify the signal.<sup>14</sup> This technique has been shown to increase the sensitivity for in situ detection of antigens and DNA markers in different systems.<sup>15</sup>

“Our results suggest that the sensitivity of culture, even after testing multiple isolates, to identify strains with different genotypes is lower than that of in situ hybridisation”

A recent study reported the ability of cag PAI (+) strains to colonise the intercellular space of epithelial cells in culture and to disrupt the organisation of tight junctions.<sup>16</sup> In our

study, we found that in cases of colonisation with cagA(-) strains, infection is mostly seen in the mucous gel or in areas near the apical surface of epithelial cells, whereas in cases of colonisation with cagA(+) strains, the bacteria are seen in more intimate contact with the epithelium, in the immediate vicinity of the epithelial cells or in the intercellular epithelial spaces. In fact, the study of cases with infection of both cagA(+) and (-) strains allowed us to document further the areas of colonisation by these two strains. We confirm the observations made in tissue cultures with cagA(+) strains,<sup>16</sup> and suggest that colonisation by cagA(-) strains is restricted to the mucous gel or the apical surface of epithelial cells. Although this pattern of colonisation by cagA(+) and (-) strains has been suggested previously,<sup>17</sup> our results document for the first time that this pattern of colonisation occurs in vivo. We acknowledge the possibility that the patterns of colonisation seen here might not result exclusively from the presence of the cag PAI, and that other genes, such as babA, a gene encoding an adhesin with affinity for the Lewis b antigen, might also play a role.<sup>18</sup>

The method described here could help elucidate the role of cagA in the development of gastroduodenal diseases associated with *H. pylori* infection. Our results document a difference between the in vivo interaction of *H. pylori* cagA(+) or cagA(-) strains and the gastric mucosa.

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#### Take home messages

- We developed an in situ hybridisation method to localise cagA(+) and (-) strains of *Helicobacter pylori* in the stomach of infected individuals
- In cases of colonisation with cagA(-) strains, infection was seen mostly in the mucous gel or in areas near the apical surface of epithelial cells, whereas in cases of colonisation with cagA(+) strains, the bacteria were seen in more intimate contact with the epithelium, in the immediate vicinity of the epithelial cells or in the intercellular epithelial spaces
- These results confirm the observations made in tissue culture with cagA(+) strains
- This method might help elucidate the role of cagA in the development of gastroduodenal diseases associated with *H. pylori* infection

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