Helicobacter pylori cag pathogenicity island genotype diversity within the gastric niche of a single host

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cag pathogenicity island (PAI) integrity was investigated in isolates from multiple biopsies recovered from 40 patients in an attempt to determine the co-existence of a varying *cag*PAI-positive to *cag*PAI-negative ratio in a single host. Six biopsies were obtained from each patient during the same endoscopic session. *cag*PAI analysis included amplification of seven loci (*cagA*, *cagE*, *cagG*, *cagM*, *cagT*, HP0527 and HP0524) and the left end of *cag*II (LEC). Absence of the island was confirmed by empty-site PCR. *lspA-glmM* RFLP and random amplified polymorphic DNA PCR were used for strain delineation. The number of biopsies with *Helicobacter pylori*-positive culture ranged from three to six per patient and a total of 218 isolates were recovered. Mixed infection was only found in two patients. Nearly one-third of the 40 patients harboured isolates with an intact *cag*PAI in all niches, another third of the isolates were empty-site-positive in all niches, whilst the remaining third of the isolates had a disrupted *cag*PAI in all or at least one of the niches. Co-existence of variants of the same strain with different *cag*PAI genotypes was observed in one-quarter of patients. The variations in *cag*PAI genotype included co-existence of: diverse *cag*PAI deletions in different niches, variants with intact and with partially deleted islands, variants with empty-site-positive and with partially deleted *cag*PAIs, and variants with an intact *cag*PAI and with empty-site-positive. Half of the patients with different *cag*PAI genotypes harboured an intact *cag*PAI in at least one niche. Co-existence of diverse genotypes of putative virulence factors in a single host must be considered when drawing a correlation with clinical presentation.

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INTRODUCTION

Among *Helicobacter pylori* virulence factors, the *cag* pathogenicity island (PAI) has been shown to be involved in inducing inflammation, ulceration and carcinogenesis (Rohde *et al.*, 2003; Hatakeyama, 2004; Shibata *et al.*, 2005). The *cag*PAI is a 37 kb region containing 28 putative genes encoding proteins involved in a specialized type IV secretion system related to the translocation of the immunodominant CagA protein into gastric epithelial cells and to the triggering of signalling cascades that lead to proinflammatory cytokine release (Fischer *et al.*, 2001; Naumann, 2005). However, the island is not a uniformly conserved entity, and is prone to disruption due to various genetic rearrangements occurring inside and outside the

constituent genes (Jenks *et al.*, 1998). According to Kauser *et al.* (2004), the *cag*PAI is highly conserved in Japanese isolates, least conserved in European and African isolates, and very poorly conserved in Peruvian and Indian isolates. These authors found that deletion frequencies of the *cagA*, *cagE* and *cagT* genes were at their highest in benign cases, whereas the *cagA* promoter and the left end of the *cag*PAI (LEC) were frequently rearranged in isolates from severe cases. However, Hsu *et al.* (2002) noted that clinical presentation could not be predicted by *cagA*, *cagE*, *cagG*, *cagM*, *cagT*, *orf13* (HP0524) and *orf10* (HP0527) genes or by the presence of an intact *cag*PAI. The lack of correlation of different *cag*PAI rearrangements with clinical presentation has also been noted in several other reports (Sheu *et al.*, 2002; Kawamura *et al.*, 2003; Nishiyama *et al.*, 2004). Nilsson *et al.* (2003) found that an intact *cag*PAI was associated with a fivefold increased risk of severe disease outcome and suggested that isolates with internal deletions had reduced

Abbreviations: LEC, left end of the *cag*PAI; PAI, pathogenicity island; RAPD, random amplified polymorphic DNA.

virulence comparable to those that were *cagPAI* negative. Therefore, the association of the *cagPAI*, as a whole or in part, with clinical presentation is not yet completely understood.

It is known that initial colonization of the gastric mucosa by a founder *H. pylori* strain leads to persistent infection (Marshall *et al.*, 1998). Over a period of time, bacterial subpopulations with highly similar genomes colonizing different regions of the stomach can emerge (Marshall *et al.*, 1998; Blaser & Berg, 2001). Rearrangement of the *cagPAI* appears to be a prevalent phenomenon, and its constituent genes could be under more selection pressure than other regions in the chromosome (Kausar *et al.*, 2004). Thus a varying ratio of *cagPAI*-positive to *cagPAI*-negative isolates could co-exist within a single host as a way of avoiding host constraints and ensuring *H. pylori* persistence. In an attempt to demonstrate this co-existence, PCR-based genotyping approaches were used to investigate *cagPAI* integrity in paired isolates recovered from multiple biopsies of single hosts.

METHODS

Patients. The study included 40 patients with positive *H. pylori* cultures from 118 consecutive patients referred to the Division of Gastroenterology of The Clinical University Hospital 'Don José de San Martín' and to the Endoscopy Service of Hospital Juan A. Fernández for gastroscopy on clinical grounds. All patients agreed to participate in the study by signing an informed consent form. Patients ranged from 18 to 92 years old (mean 49 years) and their ethnic origins were 24 European, four American Indian and 12 racially mixed. Twenty-six patients had superficial gastritis, nine had gastric erosion and five had gastric ulceration.

Six biopsy specimens were obtained from each patient using FB-24KR Olympus biopsy forceps: A1, from the mid greater curvature of the antrum; A2, from the greater curvature facing the incisura angularis; A3, from the antral lesser curvature within 2 cm of the pylorus; C1, from the middle portion of the greater curvature of the corpus; C2, from the greater curvature within 3 cm proximal to the antral–corpus border; and C3, from the lesser curvature within 3 cm of the Z line.

Bacterial culture and DNA extraction. All biopsies were cultured on blood agar base no. 2 (Oxoid) containing 7% (v/v) defibrinated horse blood with triphenyltetrazolium sodium salt (40 µg ml⁻¹) and sodium pyruvate (0.025%), and also supplemented with vancomycin (10 µg ml⁻¹) and amphotericin B (5 µg ml⁻¹). Plates were incubated at 37 °C under microaerophilic conditions for up to 5 days. DNA was extracted from confluent cultures with fewer than three *in vitro* passages by standard protocols, using SDS, lysozyme and proteinase K, followed by phenol/chloroform extraction and ethanol precipitation.

Strain genotyping and *cagPAI* PCR analysis. *lspA-glmM* RFLP using *AluI* and *HhaI* (Leanza *et al.*, 2004) and random amplified polymorphic DNA (RAPD)-PCR using primer A04 (5'-ATC-AGCGCACCA-3'; Konno *et al.*, 2005) were performed for strain delineation. Restricted fragments were electrophoresed in a 3.5% agarose gel and RAPD-PCR products in a 1.5% agarose gel. Banding patterns were analysed visually and all loci were scored for the presence or absence of a band. Fingerprint similarities were assessed using the Dice coefficient, as described previously (Leanza *et al.*, 2004). A cut-off level of 80% similarity was considered to delineate different strains (Leanza *et al.*, 2004). Roman numerals were used to name strains determined by each genotyping method.

cagPAI PCR analysis was carried out with eight oligonucleotide pairs specific for the *cagA*, *cagE*, *cagG*, *cagM*, *cagT*, *orf10* and *orf13* genes and for LEC (Ikenoue *et al.*, 2001; Hsu *et al.*, 2002). Table 1 shows the nucleotide sequences of primers used for each gene or DNA region amplification. The absence of the *cagPAI* was confirmed by amplification of a 550 bp fragment using primers specific to a region flanking this island (empty-site PCR; Mukhopadhyay *et al.*, 2000). The PCR program comprised a 3 min pre-incubation at 95 °C,

Table 1. Primers for *cagPAI* genotyping used in this study

Gene or DNA region amplified	Primer	Primer sequence (5'→3')	Size of PCR product (bp)	Annealing temperature (°C)	Reference
<i>cagA</i>	cagAF	TTGACCAACAACCACAAACCGAAG	183	54	van Doorn <i>et al.</i> (1998)
	cagAR	CTTCCCTTAATTGCGAGATTCC			
<i>cagE</i>	cagEF	GTTACATCAAAAATAAAGGAAGCG	735	54	Hsu <i>et al.</i> (2002)
	cagER	CAATAATTTTGAAGAGTTTCAAAGG			
<i>cagG</i>	cagGF	GCCATGTTAACACCCCCTAG	497	50	Hsu <i>et al.</i> (2002)
	cagGR	TTAATGCGCTAGAATAGTGC			
<i>cagM</i>	cagMF	ACAAATACAAAAAGAAAAAGAGGC	587	55	Hsu <i>et al.</i> (2002)
	cagMR	ATTTTCAACAAGTTAGAAAAAGCC			
<i>cagT</i>	cagTF	TCTAAAAAGATTACGCTCATAGGCG	490	52	Hsu <i>et al.</i> (2002)
	cagTR	CTTTGGCTTGCATGTTCAAGTTGCC			
<i>virB10 (orf13)</i>	ORF13F	CGTTCATGTTCCATACATCTTTGGC	617	55	Hsu <i>et al.</i> (2002)
	ORF13R	GATTTATAGCGATCTAAGAAACCGC			
<i>virD4 (orf10)</i>	ORF10F	AATAGTGCTTTCTTTAGCATTAGCG	658	54	Hsu <i>et al.</i> (2002)
	ORF10R	CCGATTTAATCCTTTAGGATTAGCG			
LEC	lec1-F	ACATTTTGGCTAAATAAACGCTG	363	54	Ikenoue <i>et al.</i> (2001)
	lec1-R	TCTCCATGTTGCCATTATGCT			
<i>cagPAI</i> empty site	Luni 1	ACATTTTGGCTAAATAAACGCTG	550	54	Mukhopadhyay <i>et al.</i> (2000)
	R-5280	GGTGTCACGCATTTTCCCTTAATC			

followed by 38 cycles of 1 min at 95 °C, 1 min at the annealing temperature indicated in Table 1 and 1 min at 72 °C. Final extension was performed for 7 min at 72 °C. For each gene amplification, PCRs of isolates from different biopsies of single patients were carried out simultaneously. *H. pylori* strain 26695 DNA was used as a positive control and three DNAs from single colonies of clinical isolates that were positive for empty-site PCR were used as negative controls. PCR was performed at least twice for each sample with basically identical results.

RESULTS AND DISCUSSION

In 29 of the 40 patients (72.5 %), *H. pylori* was isolated from the six biopsies obtained from each patient. In four patients, *H. pylori* was recovered from 5/6 biopsies, in three patients from 4/6 biopsies, and in the remaining four patients from 3/6 biopsies only. Therefore, a total of 218 *H. pylori* isolates were recovered. *lspA-glmM* RFLP showed 42 distinguishable banding patterns with either *HhaI* or *AluI*. RAPD-PCR determined 37 different profiles. Fig. 1 shows the 42 different fingerprints determined by *lspA-glmM* RFLP with *HhaI* and the 37 profiles shown by RAPD-PCR. Inter-niche mixed infection was detected in only two patients. In one patient, the strain denoted *lspA-glmM* RFLP-XI was isolated from the three antral biopsies and from one biopsy from the corpus, and strain *lspA-glmM*

RFLP-XII was isolated from the remaining two corpus biopsies. All isolates from the antrum and corpus showed the same profile by RAPD-PCR (Fig. 1a, b, lanes 10 and 11). In the other patient with mixed infection, strain *lspA-glmM* RFLP-XXIII was identified in the three antral biopsies and in two of the corpus biopsies, and strain *lspA-glmM* RFLP-XXIV was recovered from the remaining corpus biopsy. All isolates were also determined by RAPD-PCR to be a single strain (Fig. 1a, b, lanes 21 and 22; Table 2, patient 9C).

According to the selected loci, isolates with intact *cagPAI* were recovered from all of the biopsies with positive culture in 13 of the 40 patients, including those isolates obtained from one patient with mixed infection. In another 13 patients, all isolates recovered from the different positive niches showed absence of the *cagPAI*.

In one patient, isolates with an intact *cagPAI* were recovered from the antrum whilst isolates showing absence of the island were recovered from the corpus. Conversely, in another patient, isolates with an intact island were identified from the corpus and isolates that were empty-site-positive from the antrum. In both patients, the *cagPAI*-positive and *cagPAI*-negative isolates were variants of the same strain (Table 2, patients 60F and 63F).

Fig. 1. *lspA-glmM* RFLP and RAPD-PCR fingerprints. Each lane in (a) and (b) shows the banding patterns of the same isolate obtained with each genotyping method. (a) *lspA-glmM* RFLP fingerprints using *HhaI* (Leanza *et al.*, 2004). Lanes: 1–6, profiles that delineated strains I–VI; 7–12, strains VIII–XIII; 13–39, strains XV–XLI; 40, strain VII; 41, strain XIV; 42, strain XLII. (b) RAPD-PCR fingerprints obtained using primer A04 (Konno *et al.*, 2005). Lanes: 1–9, profiles of strains I–IX; 10 and 11, isolates recovered from different biopsies of the same patient that exhibited an identical banding pattern by RAPD-PCR (strain X), but were classified as two different strains by *lspA-glmM* RFLP (all isolates harboured intact *cagPAI*); 12–20, strains XI–XIX; 21 and 22, isolates recovered from the same patient, classified by RAPD-PCR as strain XX (9C, Table 2) but classified as two different strains by *lspA-glmM* RFLP; 23–39, strains XXI–XXXVII; 40–42, isolates with profiles XXX, XXIX and XXIV but with different *lspA-glmM* RFLP fingerprints to the isolates in lanes 32, 31 and 26, respectively.

Table 2. Patients with disrupted cagPAI and/or different cagPAI genotypes among different niches

Patient*	No. of positive niches†	cagPAI genotype‡	lspA-glmM profile	RAPD-PCR profile	Biopsy sites§					
					A1	A2	A3	C1	C2	C3
60F	4	Intact	XVIII	XVII	+	–	–	–	–	–
		Empty site	XVIII	XVII	–	–	–	+	+	+
63F	6	Empty site	XIX	XVI	+	+	+	–	–	–
		Intact	XIX	XVI	–	–	–	+	+	+
19F	6	E, G, M	V	V	+	+	+	+	+	+
32F	6	ORF13, ORF10	IX	VIII	+	+	+	+	+	+
38C	6	A, G, M, T, ORF13, ORF10, LEC	XXXIV	XXX	+	+	+	+	+	+
37F	6	AE	X	IX	+	+	+	+	+	+
29F	6	A, E, T ORF13, ORF10, LEC	VIII	VIII	+	–	+	–	–	–
		A, T, ORF13, ORF10, LEC	VIII	VIII	–	+	–	+	+	+
35C	6	E, G, M, T, ORF13, ORF10, LEC	XXXII	XXVII	+	–	–	–	–	–
		M, T, ORF13, ORF10, LEC	XXXII	XXVII	–	+	–	–	–	–
		G, M, T, ORF13, ORF10, LEC	XXXII	XXVII	–	–	+	–	–	–
		A, M, T, ORF13, ORF10, LEC	XXXII	XXVII	–	–	–	+	+	+
52F	6	A, E, G, ORF13	XV	XII	+	–	+	–	+	–
		G	XV	XII	–	+	–	+	–	+
58F	6	A, E, G, T, ORF10, LEC	XVII	XIV	+	+	–	+	+	+
		A, E, G, ORF10, LEC	XVII	XIV	–	–	+	–	–	–
42C	6	Intact	XXXVII	XXXIII	+	+	+	–	+	+
		A, E, T, ORF13, ORF10, LEC	XXXVII	XXXIII	–	–	–	+	–	–
8C	5	Intact	XXII	XIX	+	–	–	+	–	+
		A, E, G, T, ORF13, ORF10, LEC	XXII	XIX	–	+	+	–	–	–
48C	3	Empty site	XL	XXXVI	+	–	–	–	+	–
		E, M, ORF10	XL	XXXVI	–	+	–	–	–	–
9C	6	Empty site	XXIII	XX	+	+	+	–	+	–
		Empty site/A, E, M, T, ORF13, ORF10, LEC	XXIII	XX	–	–	–	+	–	–
		Intact	XXIV	XX	–	–	–	–	–	+

*F, Patients from Juan A. Fernández Hospital; C, patients from The Clinical University Hospital. Numbers indicate the order of inclusion in the study.

†Number of biopsies (niches) with *H. pylori*-positive culture obtained from each patient.

‡Intact, intact cagPAI in terms of the loci selected for the analysis; empty site, empty-site PCR-positive amplification of the genome regions flanking the left and right end of cagPAI (absence of the entire cagPAI). A, cagA; E, cagE; G, cagG; M, cagM (the four selected loci of the cagI region of the cagPAI); T, cagT; ORF13, HP0524 (*orf13*); ORF10, HP0527 (*orf10*) (the three selected loci of the cagII region of the cagPAI, Hsu *et al.*, 2002); LEC, left end of the cagPAI (Ikenoue *et al.*, 2001).

§Site in the stomach where a specific cagPAI variant was isolated (see Methods). The presence (+) or absence (–) of a specific cagPAI variant is indicated.

For the remaining 12 patients, isolates showing partial deletion within cagPAI were recovered from all or at least from one of the different niches. From four of these, isolates with the same cagPAI rearrangement were recovered homogeneously from the antrum and corpus (Table 2, patients 19F, 32F, 38C and 37F), whereas in the remaining eight patients, different cagPAI genotypes were found in one or more niches of a single host (Table 2, patients 29F, 35C, 52F, 58F, 42C, 48C, 8C and 9C). In four of these eight patients, variants of the same strain were identified with diverse cagPAI deletions in different niches (Table 2, patients 29F, 35C, 52F and 58F). In addition, co-existence of variants with intact and partially deleted

islands in different niches was identified in two patients (Table 2, patients 42C and 8C), whilst variants of the same strain that were empty-site-PCR positive and had a partially deleted cagPAI were also found in another patient (Table 2, patient 48C). From the patient harbouring *lspA-glmM*-RFLP strains XXIII and XXIV described above, two cagPAI variants of strain XXIII were identified. From the antrum and proximal to the antral–corpus border, strain XXIII isolates were found to be empty-site-positive, whereas from the greater curvature of the corpus, co-existence of isolates with an empty site and isolates with cagPAI lacking the cagG gene were identified (Table 2, patient 9C). Both variants, recognized by expansion of

single colonies from the stored sweep of the original culture plates after PCR results, showed the presence of different *cagPAI* genes simultaneously with empty-site-positive PCR results. Strain XXIV recovered from the lesser curvature of the corpus carried an intact *cagPAI* (Table 2, patient 9C). This case could support the hypothesis that strains differing in virulence potential can colonize in a mixed infection, thus allowing recombinant variants to emerge (Kersulyte *et al.*, 1999).

With respect to *cagPAI* rearrangement, 18 different genotypes were found. Among the eight analysed loci, the most frequently deleted gene was *cagM* (10/18), whereas the least frequently deleted ones were *orf10*, *orf13* and LEC (3/18, 5/18 and 6/18, respectively). *cagA*, *cagT* and *cagE* showed the same deletion rate (7/18). The finding of a conserved LEC agrees with the report of Kauser *et al.* (2004) showing that this region was rearranged more frequently in isolates linked to severe pathology worldwide. The indication that the *cagE* gene is a good marker of an intact *cagPAI* in Japanese isolates (Ikenoue *et al.*, 2001) contrasts with our finding of simultaneous deletion of *cagA*, *cagT* and *cagE*. However, deletion of *cagA*, *cagE* and *cagT* has been reported to be more frequent in isolates linked to benign infections than in isolates recovered from patients with severe ulcers and gastric cancers (Kauser *et al.*, 2004).

The results shown in this study indicated that nearly one-third of the patients harboured isolates with an intact *cagPAI* homogeneously in all positive niches, another one-third of the isolates were empty-site-PCR positive in all positive niches and the remaining one-third had a disrupted *cagPAI* in all or at least one niche. These results may be associated with the fact that most of the patients in the study population had superficial gastritis. Nilsson *et al.* (2003) suggested that the presence of a complete *cagPAI* gives a fivefold increased risk of severe disease outcome compared with an intermediate *cagPAI*. These authors found no significant difference in the risk of developing severe disease among patients infected with *cagPAI*-negative strains and those with strains carrying the intermediate genotype. However, investigation of *H. pylori* *cagPAI* genotypes from different human populations has demonstrated that the *cagPAI* appears to be disrupted in the majority of patients worldwide, with a range of conservation from 57.1% in Japanese strains to 4–15% in European isolates (Kauser *et al.*, 2004). In this study, the finding of an intact *cagPAI* recovered homogeneously from multiple paired antrum/corpus biopsies in almost 30% of patients is high considering the European ethnic origin of the study population.

Co-existence of variants of the same strain with different *cagPAI* genotypes was observed in one-quarter of patients. This occurrence may reflect the physiological differences among gastric regions of a given host that would select for derivatives that adapted better in other available locations (Akada *et al.*, 2003). A recent study in which two paired

biopsies were analysed, one from the antrum and another from the corpus, also demonstrated the occurrence of variants of the same strain differing with respect to polymorphism of the *cagA* locus (Carroll *et al.*, 2004). The identification of isolates from a single host sharing an ancestral relationship undergoing independent genomic alterations reinforces the phenomenon termed microevolution displayed by *H. pylori* during persistent colonization (Marshall *et al.*, 1998; Blaser & Berg, 2001). Although these ten patients had superficial gastritis, no conclusions can be drawn about disease association, as only nine of the 40 patients had erosion, and just five had ulceration.

No association was found between the presence or absence of inter-niche variation and previous eradication therapy administration. Among these ten patients showing co-existence of different *cagPAI* genotypes, five harboured an intact *cagPAI* in one or several niches. Considering the multiple processes initiated or supported by *cagPAI*, such as the induction of the innate immune response, cell-cycle control, cytoskeletal reorganization, disruption of cell–cell adhesion and cell motility, it is tempting to speculate that severe disease could be the result of the cumulative effect of multiple interactions between the bacteria and its host that select a variant with high virulence potential. However, the absence of the *cagPAI* among strains from varied clinical outcomes could also denote that *cagPAI* may not be the specific virulence factor associated with disease outcome.

In conclusion, the present study demonstrated the co-existence of a varying ratio of *cagPAI*-positive to *cagPAI*-negative isolates in a single host. The presence of variants located at separate niches emphasizes the potential complexity of the gastric ecosystem (physiological differences between regions of a given host's gastric mucosa such as pH and temperature, in addition to local chemical milieu including host-defence molecules after microbial colonization) and how local differences may promote *H. pylori* genetic divergence during chronic infection. However, most of the patients in the study population harboured isolates with an intact *cagPAI*, or that were empty-site-positive, or isolates with a disrupted *cagPAI*, homogeneously in all of the niches with positive culture. Therefore, the presence or absence of variants with different *cagPAI* genotypes in a single host may be related to the genetic characteristics of both the *H. pylori* colonization founder cell and the host, and could be the result of multiple bacteria–host interactions. Nevertheless, the possibility of the co-existence of diverse genotypes of putative virulence factors in different stomach niches of a single host must be considered when drawing a correlation with clinical presentation.

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