Infection, Genetics and Evolution 16 (2013) 377–385

Contents lists available at SciVerse ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Helicobacter pylori heterogeneity in patients with gastritis and peptic ulcer disease

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ARTICLE INFO

Article history:
Received 30 June 2012
Received in revised form 25 February 2013
Accepted 28 February 2013
Available online 21 March 2013

Keywords:
*cag*PAI
oipA
vacA
bab genes
Microevolution

ABSTRACT

Genetic diversification allows *Helicobacter pylori* to persist during chronic colonization/infection. We investigated the intra-host variation of several markers that suggested microevolution in patients with chonic gastritis (CG) and peptic ulcer disease (PUD). One-hundred twenty-six isolates recovered from 14 patients with CG and 13 patients with PUD were analysed. *cag* pathogenicity island (*cag*PAI), *oipA*, *vacA*, *bab* gene status and the presence of *jhp*0926, *jhp*0945, *jhp*0947, *jhp*0949 and *jhp*0940 genes from the genomic Plasticity Zone (PZ) were taken into accout to investigate intra-host variation. *lspA-glm*M-RFLP was performed to identify mixed infections. Only one patient was colonised/infected by two ancestrally unrelated strains. Among the 126 isolates, a significant association among *cag*PAI genotypes, *oipA* status and *vacA* alleles was indicated. Complete *cag*PAI, *oipA* "on", and *vacA* s1-m1 variants were significantly found in patients with PUD, without intra-host variations. Isolates from 7/14 patients with CG lacked *babA* in all chromosomal loci. In contrast, isolates from all or several biopsies of PUD patients carried *babA*, but in one patient only, the isolates showed positive Lewis b (Leb) binding assay. Considering *cag*PAI, *vacA*, *oipA, bab* genotypes, intra-host variation was also significantly higher in patients with CG. Conversely, a similarly high intra-host variation in almost PZ genes was observed in isolates from patients with CG and PUD.

In conclusion, the lowest intra-host variation in *cag*PAI, *oipA*, *vacA*, and *bab* genes found in patients with PUD suggests the selection of a particular variant along the bacteria-host environment interplay during ulceration development. However, the predominance of this variant may be a refletion of the multifactorial etiology of the disease rather than the cause, as it was also found in patients with CG. The intra-host variation in PZ genes may predict that this genomic region and the other markers of microevolution studied evolve under diverse pressure(s).

1. Introduction

Helicobacter pylori shows exceptional genetic variability and intraspecies diversity. This genetic diversity is postulated to be required for persistent colonisation of the stomach, where different microenvironments and changing conditions are likely to be encountered (Kuipers et al., 2000; Israel et al., 2001; Björkholm et al., 2001; Kraft et al., 2006; Suerbaum and Josenhans, 2007; Ahmed, 2010). However, it has long been assumed that the status or genotype of putative virulence factors are stable characteristics and can be linked to disease progression. Several reports have presented data against these assumptions (Akhter et al., 2007; Atherton and Blaser, 2009; Ahmed, 2010) and it seems that putative virulence factor genes can evolve as a result of the bacteria-host interaction during colonisation (Kim et al., 2009; Ahmed, 2010). Microevolution can include isolates exchanging *cagA* alleles or losing all or part of the *cag* pathogenicity island (*cag*PAI) along with the infection through recombination (Kersulyte et al., 1999; Kraft et al., 2006). Inter-genomic recombination with other strains may also affect other putative virulence factors e.g. *vacA* genotype and *vacA* phenotype (Carroll et al., 2004; Atherton and Blaser, 2009). Argent et al. (2008) demonstrated that the microevolution in *cagA* and *vacA* genes was a common event within isolates recovered from families of gastric cancer patients, leading to a change in the virulence phenotype. An approach to investigate microevolution in a single host is the analysis of the genetic relationship

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http://dx.doi.org/10.1016/j.meegid.2013.02.024

among *H. pylori* isolates recovered sequentially from the same patient. This approach was used by Kraft et al. (2006) who reported differences in the *cag*PAI genotype and changes involving the Plasticity Zone of the genome, among others. Also, Alvi et al. (2007) described microevolution in *cagA*, *cagE*, and *cagY* genes of the *cag*PAI and also in *tfs*3 (type IV secretion system) in isolates obtained from one patient with duodenal ulcer at inclusion in the study and after a 10-year period. Similarly, Prouzet-Mauléon et al. (2005) analysed two successive isolates obtained 9 years apart from a duodenal ulcer patient on *H. pylori* eradication therapy. Through three different fingerprinting approaches, the authors demonstrated that the two isolates derived from the same strain, and microevolution was observed in the *cagA* gene, the *cag*PAI right junction, the *vacA* m1 allele, and in the member of the plasticity region cluster studied (JHP926). Another method to investigate this microevolution phenomenon is the analysis of multiple biopsies obtained at the same endoscopic session (paired isolates) (Carroll et al., 2004; Akhter et al., 2007). Various genotyping methods applied in paired isolates from antral and corpus biopsies revealed similar fingerprints with minor differences. These results may be possible due to the fact that isolates recovered from a patient may be ancestrally related with a founder strain but had undergone independent genomic alterations (Carroll et al., 2004; Akhter et al., 2007). Our previous studies on the analysis of paired isolates from multiple biopsies (niches) of a single host in patients with established colonisation (mean age 49 years) demonstrated microevolution of *cag*PAI in 10/38 patients colonised by a single strain. The frequency of *oipA* and *bab* genes inter-niche variability was similar to *cag*PAI, while in *vacA* it was less common (Matteo et al., 2007, 2010, 2011). On the assumption that *H. pylori* uses mutation and recombination processes to adapt to its individual host by modifying molecules that interact with the host, this microevolution during colonisation could also be linked to virulence optimization (Atherton and Blaser, 2009). Therefore, the selection of a single variant could be the result of long-term host-bacterial interactions ultimately leading to disease development. This study assessed the frequency of variants with different status of *cag*PAI, *oipA*, *vacA*, *bab*, and five genes located in the Plasticity Zone (PZ) of multiple gastric niches in patients with chronic gastritis (CG) and peptic ulcer disease (PUD), referred for upper gastric endoscopy during 2010–2011 in order to investigate the hypothesis of the prevalence of a unique or low number of variants when the diagnosis of PUD was established.

2. Material and methods

2.1. Patients and biopsies

One hundred and twenty-six isolates recovered from 175 biopsies of 27 *H. pylori* positive patients (13 with PUD, 14 with CG) were included. The 14 patients with CG were referred for upper gastric endoscopy to the Gastroenterology Service of Clínica Bazterrica, Buenos Aires, in 2010 and the 13 patients with PUD (11 patients with gastric ulcer and two patients with duodenal ulcer) were referred to the Centro Integral de Gastroenterología, Buenos Aires, in 2011. Patients shared similar socio-economic status and lifestyle. All patients agreed to participate in the study by signing an informed consent. Biopsy specimen sites were: a1, the mid greater curvature of the antrum; a2, the greater curvature facing the incisura angularis; a3, the antral lesser curvature; c1, the middle portion of the greater curvature of the corpus; c2, the greater curvature within 3 cm to the antral–corpus border; c3, the lesser curvature within 3 cm of the Z line; U, the ulcer edge, obtained at the same endoscopic session. All biopsies were cultured as described previously (Matteo et al., 2007).

2.2. DNA extraction and strain delineation

DNA was extracted from confluent cultures in line with standard protocols using a pool of colonies from the isolation plate of each niche (biopsy) and from the subculture of a single colony of this plate. In order to identify mixed infections of the gastric mucosa, the combination of *lsp*A-*glm*M RFLP digested with *Alu*I (Kersulyte et al., 1999), *Hha*I (Han et al., 2000) and *Sau*3A1 (Han et al., 2000; Kivi et al., 2003,) was used for strain delineation. A total of 61 bands were scored by combining the restriction profiles of the three enzymes.

2.3. Microevolution of putative disease markers

To investigate intra-host variation in *cag*PAI, the presence or absence of *cagA, cagE, cagG, cagM, cagT, cagY*, and *cagβ* genes was considered. Three different sets of primers were used for the PCR amplification of *cagA, cagE, cagG, cagM* and *cagT*, and four pairs were employed to detect *cagY* and *cagβ* genes (Table S1). The absence of *cag*PAI was confirmed by the amplification of a 550-bp fragment with primers specific to the regions flanking the genomic island (empty-site PCR) (Matteo et al., 2007). PCR was performed simultaneously on each *cag*PAI gene of isolates from different biopsies of a single patient. *Helicobcter pylori* strain 26695 DNA was used as a positive control, and three DNA from single colonies of clinical isolates, positive for empty-site PCR, were used as negative controls. PCR was performed at least twice on each sample with basically identical results. *oipA* status was investigated by the amplification of the 5́ region of the gene using the set of primers shown in Table S1. PCR products were purified using Wizard-PCR Preps (Promega, Madison, WI, USA), according to the manufacturer's instructions. Sequencing was performed on both strands using an ABI 373 DNA sequencer (Applied Biosystems, SA, Argentina). For the *oipA* microevolution, the number of CT repeats at the 5′ region of the gene was classified into an "on" or "off" status, according to Yamaoka et al. (2002), also taking into account that in several isolates, TT and CC can separate the number of CT repeats in two groups (Matteo et al, 2010). *vacA* alelles s1, s2, m1 and m2 were performed by multiplex-PCR as described previously (Leanza et al., 2004). Presence or absence of the PZ genes *jhp*0926, *jhp*0945, *jhp*0947, *jhp*0949 and *jhp*0940 was investigated by PCR employing two different pairs of primers for each of them (Table S1). In the 71 isolates from PUD patients, *bab* genes were investigated by PCR at the common three chromosomal sites using consensus primers with *hpyD* (locus A) and *s18* (locus B), combined with primers for *babA* and *babB* (Colbeck et al., 2006); LC-F1 and LC-R1 primers were used to analyse locus C (Hennig et al., 2006), and fragments of 500–3000 bp were considered empty sites. *babA*, *babB* or *babC* at each locus and locus C empty-site were confirmed by sequence analysis. The *bab* gene promoter region was amplified for the sequence analysis with one of the following primers: LocA-1 (5́GGCTCATAACCCAAAGGTC-3́), LocA-2 (5́-GTTTTG GTCCTGGCATTC-3́), LocB-1 (5́-GGATAGCCCTTTAAAACAGC-3́), or LocB-2 (5́-GAAGTAGCGA TCAAAAGAG- 3́) and with the *babA* and *bab*B specific primers (Matteo et al., 2011). Purification of PCR amplicons and sequencing were performed as described previously. *bab* genes in the 54 isolates from CG patients were studied previously, among other isolates from patients without PUD (Matteo et al., 2011). The remaining markers were investigated in the 126 isolates included in this study.

The *oipA* sequences studied here were assigned the following GenBank accession numbers JF891401-JF891415, JX122410-JX122425, and *bab* sequences JF922351-JF922380.

2.4. Binding to Lewis b conjugate

Adherence to Lewis b (Leb) antigen was assessed in the 126 isolates by ELISA using fresh isolates labelled with digoxigenin (DIG)

(Roche Diagnostics, Germany) according to Aspholm et al. (2006). Universal Covalent 96-well microtiter-plates (Microlon, Greiner bio-one, Germany) were coated with Leb-human serum albumin conjugate (IsoSep AB, Stockholm, Sweden). Detection was performed using anti-DIG-HRP-antibody and ABTS-solution (Roche Diagnostics). Extinction was quantified at 405 nm and normalized to controls (uncoated wells). In each single experiment, all isolates were tested in two coated wells, and two control isolates were also included. At least three independent assays were performed on each isolate. Isolates were considered positive if the ratio of $Ex_{Lewis}/Ex_{control}$ was >1.5.

2.5. Statistical analysis

The χ^2 test or Fisher's exact test carried out by SPSS version 20, were used to compare the differences among groups. p values <0.05 were considered significant.

3. Results

The combination of *lsp*A-*glm*M RFLP profiles obtained with *Alu*I, *Hha*I and *Sau*3A1 classified the 126 isolates in 28 distinguishable banding patterns (Fig. 1). Isolates with different banding patterns in a single host were observed in patient 16B, only. This patient carried the strain with the banding pattern I/IV/II in antrum and the strain with the banding pattern IV/V/II in corpus biopsies (Fig. 1, lanes 4 and 5)

Eighty-three (65.8%) out of the 126 isolates harboured the seven selected markers of *cag*PAI, 34 (26.9%) isolates lacked the island, and in the remaining 9 isolates (7.3%), one or more of the selected genes were not detected by the different pairs of primers used (Tables 1 and 2). Seven (50.0%) out of the 14 patients with CG showed intra-host variation of *cag*PAI genotypes. In four of these patients, the coexistence of isolates carrying the seven selected genes of *cag*-PAI and empty-site positive isolates was observed. The other three patients with CG and intra-host variation showed occurrence of isolates with all the *cag*PAI genes studied and isolates with one, two or three of these genes undetected (Table 1). Two out of the seven patients without intra-host variation harboured empty-site positive isolates and the other five possessed isolates with the seven selected markers of *cag*PAI. One of the latter five patients was that numbered 16B mentioned above (Table 2). Conversely, in the 13 patients with PUD, intra-host variation of the *cag*PAI genotype was not observed (Fisher's exact test, two tails, $p < 0.005$). Nine (69.2%) out of the 13 patients with PUD harboured isolates with all *cag*PAI selected markers and 4/13 (30.8%) patients carried empty-site positive isolates (Tables 1 and 2).

Considering the 5′ region of the *oipA* gene, the frequency of inter-host variation in the number of CT repeats was not significantly different among patients with GC and PUD (4/14 versus 1/13) (Table 1). The variation of the "on" and "off" status among intra-host isolates was observed in only one patient with CG (Table 1). Considering the 126 isolates, a significant association of the *cag*PAI genotype with *oipA* status was detected. Seventy-six (92.6%) out of 82 isolates with the *oipA* "on" status showed the presence of the seven selected markers for *cag*PAI, and 50 (65.8%) of them were recovered from patients with PUD ($p < 0.05$).*vacA* alleles showed microevolution only in three patients with CG, with intra-host coexistence of isolates *vacA* s1-m1 and *vacA* s2-m2 (Table 1). Both *vacA* alleles were similarly distributed among the isolates of patients with CG and PUD. However, a significant association was also observed among *cag*PAI genotypes, *oipA* status and *vac* alleles. Fifty-two (82.5%) out of the 63 isolates *vacA* s1-m1 showed the seven *cag*PAI selected markers and the *oipA* "on" status. Thirty-three of them were recovered from patients with PUD ($p < 0.05$). Similarly, 30 (88.2%) out of the 34 empty site positive isolates were associated with the *oipA* "off" status and the *vacA* s2-m2 allele.

Three major differences can be highlighted among *bab* genes from isolates of CG and PUD patients. These differences were: (a) highest number of isolates with the absence of *babA* in all niches in patients with CG; (b) highest number of isolates with the presence *babA*/*B* chimeric allele in several or all niches in patients with CG; and (c) prevalence of *babA* at locus A in isolates of all niches in patients with PUD (Tables 1 and 2). Microevolution in a single patient with CG included the coexistence of isolates with: (a) presence and absence of the chimeric *babA*/*B* or *babB*/*A* variants, (b) single and double copy of *bab*B, (c) presence and absence of *babC* at locus A; and (d) *bab*B gene with and without ATG translational start codon (Table 1). On the other hand, in patients with PUD microevolution included mainly the occurrence of isolates with (a) single or double copy of *babA*, (b) inverse location of *babA* and *babB*, and (c) presence and absence of *babA*/*B* (Table 1). Considering the 126 isolates, 50 (53.7%) out of 93 isolates harbouring *babA* showed the seven genes of *cag*PAI, and *oipA* "on" studied. Thirty-two (64.0%) of these 50 isolates were recovered from patients with PUD (Tables 1 and 2). However, the number of positive Leb binding assays among isolates harbouring *bab*A recovered from patients with CG was significantly higher than isolates from patients with PUD (7/23 versus 3/70, ($p < 0.05$) (Tables 1 and 2).

Similar inter-host diversity in the *babA* promoter region was observed among patients with and without PUD, with the poly-adenine tract located within the −10 to −35 ranging from nine to 18 nucleotides, and the Shine-Dalgarno sequences and ATG start codon varied in range from three to eight repeats.

In relation to *cag*PAI, *vacA*, *oipA* and *bab* genotypes, an intra-host variation was observed in one or more of these markers in 12/14 patients with CG versus 2/13 patients with PUD ($p < 0.05$) (Table 1).

The prevalence of *jhp*0926 *jhp*0945, *jhp*0947, *jhp*0949 and *jhp*0940 genes in 126 isolates was 30.1% (n: 38), 69.0% (n: 87), 45.2% (n: 57), 55.6% (n: 70), and 74.6% (n: 94), respectively. *jhp*0945, *jhp*0947, *jhp*0949, and *jhp*0940 genes were significantly prevalent in isolates from patients with CG ($p < 0.05$) (Tables 1 and 2). The intra-host variation of the presence of *jhp*0947, *jhp*0949 and *jhp*0940 genes was similar among patients with CG and patients with PUD. In contrast, *jhp*0926 and *jhp*0945 genes intra-host variation was significantly higher in isolates recovered from patients with PUD ($p < 0.05$).

The intra-host variation in one or more of all the markers studied was observed in 23 out of 27 patients (Table 1).

4. Discussion

The ability of *H. pylori* to persist within hosts that recognize its presence indicates important adaptations, undoubtedly involving programmed cross-talk between microbe and host (Hooper and Gordon, 2001). At this point, the innate and adaptive immune defenses of the host, combined with factors in the environment of the stomach, apparently drive a continuously high rate of genomic variation in *H. pylori* (Dorer et al., 2009). The coexistence of isolates harbouring *cag*PAI and empty-site positive isolates in the same host has been shown earlier by Kersulyte et al. (1999). Kraft et al. (2006) described differences also in the *cag*PAI genotype among paired isolates obtained sequentially from a single host. These authors denoted that the isolate recovered first carried the *cag*PAI, and that the second one lacked the entire island along with one of the two imperfect copies of the 31-bp repeat flanking sequences; whereas sequences upstream and downstream of the remaining copy of the repeat were identical in both isolates. This observation suggests a deletion by intrachromosomal recombination rather than by

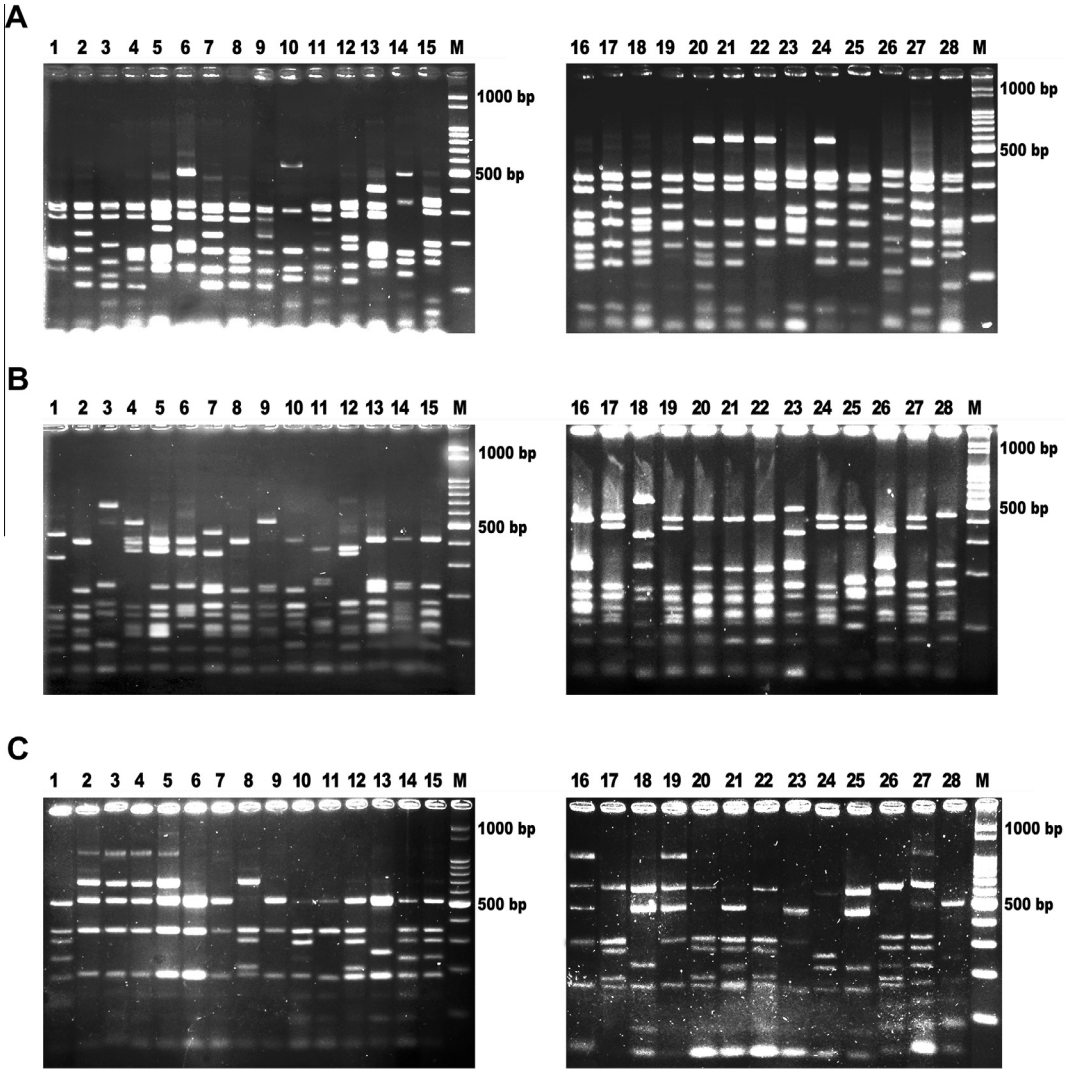

Fig. 1. *lspA-glmM* polymorphism. A AluI restriction profiles: Lanes 1, 4 and 28, type I; lanes 2, 7, 9, 17, 19, 25, 26 and 27, type II; lanes 3 and 12, type III; lane 5, type IV; lanes 6 and 22, type V; lane 8, type VI; lane 10, type VII; lane 11, type VIII; lane 13, type IX; lane 14, type X; lane 15, type XI; lanes 16 and 18, type XII; lane 20 and 21 type XIII; lane 23, type XIV; lane 24, type XV. B. HhaI profiles: Lane 1, type I; lane 2, type II; lane 3, type III; lane 4, type IV; lane 5, type V; lane 6, type VI; lane 7, type VII; lanes 8, 15, and 26, type VIII; lane 9, type IX; lane 10, type X; lane 11, type XI; lane 12, type XII; lane 13, type XIII; lane 14, type XIV; lane 16, type XV; lane 17, 19, 24 and 27, type XVI; lane 18, type XVII; lanes 20, 21 and 22 type XVIII; lane 23, type XIX; lane 25, type XX; lane 28, type XXI. C. Sau3A1 profiles: Lane 1, type I; lanes 2, 3, 4, 5, 16 and 19, type II; lanes 6, 7, 9, 11, 23 and 28, type III; lanes 8, 17, 20, 22 and 26, type IV; lanes 10, 12 and 21, type V; lane 13, type VI; lanes 14 and 15, type VII; lanes 18 and 25, type VIII; lane 24, type IX; lane 27, type X. M. DNA ladder 100 bp. Strains are delineated by the combination of the three restriction enzymes profiles. Isolates showing each combined banding patterns are indicated in Tables 1 and 2.

allelic replacement after import of an empty-site allele from an unrelated strain. In the present study, the coexistance of *cag*PAI positive and *cag*PAI negative isolates in a single host was also shown in paired isolates recovered from antrum and corpus biopsies obtained from patients with CG. Concerning the *cag*PAI rearrangement, Kauser et al. (2004) found that *cag*PAI appeared to be disrupted in the majority of isolates from patients worldwide. When comparing *cag*PAI sequences, Olbermann et al. (2010) asserted that the gene content and order were highly conserved. However, certain variations of the general gene arrangement were described, e.g.: deletion of half of the *cag*PAI, integration of IS elements at distinct locations of the island, inversion of *cagQ,* splits of *cag*PAI between *cagQ* and *cagP* into two segments, duplication of *cagA* and *cagB*, as well as more complex rearrangements comprising all *cag* genes except for *cagA* among other genetic events have been described (Olbermann et al., 2010; Fischer, 2011; Farnbacher et al., 2010). We evaluated the *cag*PAI status by the detection of seven *cag* genes and by this same approach and we found that only nine out of the 94 empty-site negative isolates showed absence of one or more of the selected genes. The isolates with disrupted *cag*-PAI genotypes were recovered from patients that also harboured isolates with all *cag*PAI selected markers, and this microevolution was observed in patients with CG only. In addition to the intra-host variation of *cag*PAI genotype, our finding revealed a significant association of a particular *cag*PAI genotype, with a specific *oipA* status and *vacA* allele. All this highlights the fact that these factors interact with each other during persitent colonisation or disease development (Dossumbekova et al., 2006; Yamaoka, 2010). There is no obvious common genetic mechanism controlling *cag*PAI, *oipA* and *vacA* genes expression, and their statuses seem to be based on the selection in the host (Dossumbekova et al., 2006). To date, *H. pylori* disease-specific genes have not been found and the fact that only a fraction of the colonised individuals ever develop diseases infers that the interaction between bacterial factors and host epithelial and immune cells plays an important role in the development of gastric injuries (Israel and Peek, 2010). During the bacteria-host

Table 1
Patients showing intra-host variation in the selected markers.

Patients[a]	No of niches	Biopsy sites[a]	*cag*PAI genes							*vacA*	*bab* genes			Binding Assay	*oipA*[d]	Plasticity zone genes					lspA-glmM profiles		
			cagA	*cagE*	*cagG*	*cagM*	*cagT*	*cagY*	*cagβ*		Locus A	Locus B	Locus C			*jhp*0926	*jhp*0945	*jhp*0947	*jhp*0949	*jhp*0940	*Alu* I	*Hha* I	*Sau*3A1
4B	2	a3	+	+	+	+	+	+	+	s2m2	*babA/B*	*babB*	empty[c]	–	off8	+	+	–	+	+	I	I	I
		c1	empty[b]							s2m2	*babA/B*	*babB*	empty	–	off7R	–	+	+	+	–	I	I	I
6B	5	a1	+	+	+	+	+	+	–	s1m1	*babB*	*babB*[h]	empty	–	on(3 + 2)[f]	–	+	–	+	+	II	II	II
		a2	+	+	+	+	+	–	+	s1m1	*babB*	*babB*	empty	–	on(3 + 2)	–	–	–	+	–	II	II	II
		c1	+	+	+	+	–	–	+	s1m1	*babB*	*babA*	empty	+	on(3 + 2)	–	–	–	+	–	II	II	II
		c2	+	+	+	+	–	+	–	s1m1	*babB*	*babB*	empty	–	on(3 + 2)	–	+	+	+	+	II	II	II
		c3	+	+	+	+	+	+	+	s1m1	*babB*	*babB*	empty	–	on(3 + 2)	–	+	+	+	–	II	II	II
15B	5	a2	+	+	+	+	+	+	+	s1m1	*babA/B*	*babA*[i]	*babA/B*	–	on6	–	+	+	+	+	III	III	II
		a3. c1–c3	+	+	+	+	+	+	+	s1m1	*babA/B*	*babA*	*babA/B*	–	on6	–	+	+	+	–	III	III	II
17B	3	a1, a3,	+	+	+	+	+	+	+	s2m2	*babA/B*	*babA/B*[j]	empty	–	off8R	–	+	+	+	–	V	VI	III
		c1	empty							s2m2	*babA/B*	*babA/B*	empty	–	off8R	–	+	+	+	+	V	VI	III
25B	3	a2	empty							s1m1	*babA*	*babB*	empty	–	on6R	–	+	–	+	+	II	VII	III
		c1	empty							s1m1	*babA*	*babB*	empty	–	on6R	–	+	+	+	+	II	VII	III
		c2	+	+	+	+	+	+	+	s1m1	*babA*	*babB*	empty	–	on6R	–	+	+	+	+	II	VII	III
24B	4	a1	+	+	+	+	+	+	+	s1m1	*babA*	*babB*	empty	+	on6R	–	+	–	+	–	VI	VIII	IV
		a3, c3	+	+	+	+	+	+	+	s1m1	*babA*	*babB*	empty	+	on6R	–	+	+	+	+	VI	VIII	IV
		c2	+	+	+	+	+	+	+	s1m1	*babA/B*, *babA*[g]	*babB*	empty	(–)/(+)[k]	on6R	–	+	+	+	+	VI	VIII	IV
26B	4	a1–a3	empty							s1m1	*babA/B*	*babB*	empty	–	off7R	+	+	+	+	+	II	IX	III
		c1	empty							s2m2	*babA/B*	*babB*	empty	–	off9R	+	+	+	+	–	II	IX	III
32B	6	a2	+	+	+	+	+	+	+	s2m2	*babB*[e]	*babB*	empty	–	on9R	–	+	+	+	–	VII	X	V
		a1,a3, c1–c3	+	+	+	+	+	+	+	s1m1	*babB*	*babB*	empty	–	on9R	–	+	+	+	+	VII	X	V
39B	2	c2	empty							s1m1	*babA*	*babB*	empty	–	off11R	–	+	–	+	+	VIII	XI	III
		c3	+	+	+	+	+	+	+	s1m1	*babA*	*babB*	empty	–	off11R,on9R[g]	–	+	+	+	+	VIII	XI	III
41B	4	a2	empty							s2m2	*babC*	*babB*	empty	–	off4(2 + 2)R	+	+	+	+	+	III	XII	V
		a3, c2, c3	empty							s2m2	*babB*	*babB*	empty	–	off4(2 + 2)R	+	+	+	+	+	III	XII	V
45B	3	a3	+	+	+	+	+	+	+	s2m2	*babA*	*babB/A*	empty	+	off8R	–	+	–	+	+	IX	XIII	VI
		c1	+	+	+	+	+	+	+	s1m1	*babA*	*babB/A*	empty	+	off8R	–	+	–	+	+	IX	XIII	VI
		c3	+	+	–	–	+	+	–	s1m1	*babA*	*babB/A*	empty	+	off8R	–	+	–	+	+	IX	XIII	VI
22	5	a1, c1	+	+	+	+	+	+	+	s2m2	*babC*	*babA*	empty	–	on6R	–	+	–	+	+	X	XIV	VII
		a2	+	+	+	+	+	+	+	s2m2	*babC*	*babA*	empty	–	on6R	+	+	–	–	+	X	XIV	VII
		c2	+	+	+	+	+	+	+	s2m2	*babC*	*babA*	empty	–	on6R	–	+	–	–	+	X	XIV	VII
		c3	+	+	+	+	+	+	+	s2m2	*babC*	*babA*	empty	–	on9R	–	+	–	–	+	X	XIV	VII
29	6	a1,a3	+	+	+	+	+	+	+	s2m2	*babB*	*babB*	empty	–	off7R	–	+	–	–	–	XI	VIII	VII
		a2, c1,c3	+	–	–	–	+	+	+	s2m2	*babB*	*babB*	empty	–	off7R	–	–	–	–	–	XI	VIII	VII
		c2	+	–	–	–	+	+	+	s2m2	*babB*	*babB*	empty	–	off7R	–	+	–	–	–	XI	VIII	VII
12C	6	a1, a2	empty							s2m2	*babA*	*babB*	empty	–	off9(2 + 7)R	–	–	–	+	+	II	XVI	IV

(*continued on next page*)

Table 1 (*continued*)

Patients[a]	No of niches	Biopsy sites[a]	*cag*PAI genes							*vacA*	*bab* genes			Binding Assay	*oipA*[d]	Plasticity zone genes					lspA-glmM profiles		
			cagA	*cagE*	*cagG*	*cagM*	*cagT*	*cagY*	*cagβ*		Locus A	Locus B	Locus C			*jhp*0926	*jhp*0945	*jhp*0947	*jhp*0949	*jhp*0940	*Alu* I	*Hha* I	*Sau*3A1
		a3	empty[b]							s2m2	*babA*	*babB*	empty	–	off9(2 + 7)R	+	–	–	+	+	II	XVI	IV
		c1–c3	empty							s2m2	*babA*	*babB*	empty	–	off9(2 + 7)R	–	–	–	–	+	II	XVI	IV
15C	6	a1	+	+	+	+	+	+	+	s1m1	*babA*	*babB*	empty	–	on 6 R	+	–	–	–	+	XII	XVII	VIII
		a2, a3, c1–c3	+	+	+	+	+	+	+	s1m1	*babA*	*babB*	empty	–	on 6 R	–	–	–	–	+	XII	XVII	VIII
16C	6	a1	+	+	+	+	+	+	+	s1m1	*babA*	*babB*	empty	–	on (5 + 2)R	+	+	–	+	–	II	XVI	II
		a2, a3, c1	+	+	+	+	+	+	+	s1m1	*babA*	*babB*	empty	–	on (5 + 2)R	+	+	+	+	–	II	XVI	II
		c2	+	+	+	+	+	+	+	s1m1	*babA*	*babB*	empty	–	on (5 + 2)R	+	+	–	+	+	II	XVI	II
		c3	+	+	+	+	+	+	+	s1m1	*babA*	*babB*	empty	–	on (5 + 2)R	+	–	–	–	–	II	XVI	II
20C	6	a1, a2, c1, c2,	empty							s2m2	*babA*	*babB*	empty	–	off9(2 + 7)R	–	–	–	+	+	XIII	XVIII	IV
		a3	empty							s2m2	*babA*	*babB*	empty	–	off9(2 + 7)R	–	–	–	+	–	XIII	XVIII	IV
		c3	empty							s2m2	*babA*	*babB*	empty	–	off9(2 + 7)R	–	+	–	+	+	XIII	XVIII	IV
43C	6	a1	+	+	+	+	+	+	+	s2m2	*babA*	*babB*	empty	–	on (5 + 2)R	–	–	–	–	+	XIII	XVIII	V
		a2, c1,c2	+	+	+	+	+	+	+	s2m2	*babA*	*babB*	empty	–	on (5 + 2)R	–	+	–	–	+	XIII	XVIII	V
		a3	+	+	+	+	+	+	+	s2m2	*babA*	*babB*	empty	–	on (5 + 2)R	+	+	+	–	+	XIII	XVIII	V
		c3	+	+	+	+	+	+	+	s2m2	*babA*	*babB*	empty	–	on (5 + 2)R	+	+	–	–	+	XIII	XVIII	V
49C	6	a1,a2, c1, u	+	+	+	+	+	+	+	s2m2	*babA*	*babB*	empty	–	on6R	–	–	+	–	+	V	XVIII	IV
		c2	+	+	+	+	+	+	+	s2m2	*babA*	*babB*	empty	–	on6R	–	–	–	–	–	V	XVIII	IV
		c3	+	+	+	+	+	+	+	s2m2	*babA*	*babB*	empty	–	on6R	–	+	+	–	–	V	XVIII	IV
6G	4	a1	+	+	+	+	+	+	+	s1m1	*babA*	*babB*	empty	+	on (1 + 4)R	+	–	–	–	+	XV	XVI	IX
		a2	+	+	+	+	+	+	+	s1m1	*babA*	*babB*	empty	+	on (1 + 4)R	–	–	–	–	+	XV	XVI	IX
		a3	+	+	+	+	+	+	+	s1m1	*babA*/*B*	*babB*	empty	–	on9R	+	+	–	–	+	XV	XVI	IX
		u	+	+	+	+	+	+	+	s1m1	*babA*	*babB*	empty	+	on9R	–	–	+	–	+	XV	XVI	IX
8G	6	a1, a2	+	+	+	+	+	+	+	s1m1	*babA*	*babB*	empty	–	on (5 + 2)R	–	–	–	–	+	II	XX	VIII
		c1, c2	+	+	+	+	+	+	+	s1m1	*babA*	*babB*	empty	–	on (5 + 2)R	+	+	–	–	+	II	XX	VIII
		c3	+	+	+	+	+	+	+	s1m1	*babA*	*babB*	empty	–	on (5 + 2)R	–	+	–	–	+	II	XX	VIII
		u	+	+	+	+	+	+	+	s1m1	*babA*	*babB*	empty	–	on (5 + 2)R	+	+	+	–	+	II	XX	VIII
13G	5	a3	+	+	+	+	+	+	+	s1m1	*babA*	*babB*	empty	–	on 6 R	–	+	–	–	+	II	VIII	IV
		c1	+	+	+	+	+	+	+	s1m1	*babA*	*babB*	empty	–	on 6R	+	+	–	–	+	II	VIII	IV
		c2, c3	+	+	+	+	+	+	+	s1m1	*babA*	*babB*	empty	–	on 6R	–	+	–	–	+	II	VIII	IV
		u	+	+	+	+	+	+	+	s1m1	*babA*	*babA*, *babB*[f]	empty	–	on 6R	–	+	–	–	–	II	VIII	IV
12F	5	a1	+	+	+	+	+	+	+	s2m2	*babB*	*babA*	empty	–	on 6R	+	–	–	–	–	I	XXI	III
		a2	+	+	+	+	+	+	+	s2m2	*babA*	*babB*	empty	–	on 6R	+	–	–	–	–	I	XXI	III
		c1	+	+	+	+	+	+	+	s2m2	*babB*	*babA*	empty	–	on 6R	+	+	–	+	–	I	XXI	III
		c3, u	+	+	+	+	+	+	+	s2m2	*babB*	*babA*	empty	–	on 6R	–	+	–	–	–	I	XXI	III

[a] 4B-29, patients with CG; 12C-12F, patients with PUD (43C and 49C duodenal ulcer disease). a1–a3, isolates from anthrum biopsies, c1–c3, isolates from corpus biopsies, u: ulcer edge biopsies.
[b] Empty: empty-site PCR-positive amplification of the genome regions flanking the left and right end of *cag*PAI (absence of the entire *cag*PAI).
[c] Empty: *babA, babB* or *babC* were not identified in locus C.
[d] R: number of CT repeats at the 5́region of the gene.
[e] Isolates from a1, a3, c1 and c3 showed *bab*B with *bab*A promoter region at locus A and with *bab*C promoter region at locus B. After ATG start codon *bab*B sequences at loci A and B are identical except for the CT repeat numbers. In addition, in isolates from c2, *bab*B at locus A lacked ATG start codon (Matteo et al., 2011).
[f] The number of CT repeats was separated in two groups by the insertion of TT or CC.
[g] Intra-niche variation, two populations of isolates in the same niche (biopsy).
[h] In locus B, inter-niche variation of the number of *bab*B CT repeat was observed.
[i] Truncated protein in all niches.
[j] *bab*A/B in locus A, promoter region with highest identity with *bab*A promoter, in locus B highest identity with *bab*B promoter. After ATG start codon, *bab*A/B sequences in both (Matteo et al., 2011).
[k] Lewis b binding assay positive for isolates with *bab*A in locusA, only.

Table 2
Patients without intra-host variation in the selected markers.

Patients[a]	No of niches	Biopsy sites[a]	*cag*PAI genes							*vacA*	*bab* genes			Binding Assay	*oipA*[d]	Plasticity zone genes					lspA-glmM profiles		
			cagA	*cagE*	*cagG*	*cagM*	*cagT*	*cagY*	*cagβ*		Locus A	Locus B	Locus C			*jhp*0926	*jhp*0945	*jhp*0947	*jhp*0949	*jhp*0940	*Alu* I	*Hha* I	*Sau*3A1
16B	3	a3	+	+	+	+	+	+	+	s1m1	*babB*	*babB*	empty [c]	–	on6R	–	–	–	+	+	I	IV	II
		c2, c3	+	+	+	+	+	+	+	s1m1	*babB*	*babB*	empty [c]	–	on6R	–	–	–	+	+	IV	V	II
1G	3	a1, a2, a3	empty [b]							s2m2	*babB*	*babA*	empty	–	off8R	+	+	+	+	+	XIV	XIX	III
15G	6	a1–a3, c1, c2, u	empty							s2m2	*babA*	*babB*	empty	–	off8R	+	+	+	+	+	II	XVI	X
4C	6	a1–a3, c2, c3, u	+	+	+	+	+	+	+	s1m1	*babA*	*babB*	empty	–	on9R	–	+	–	+	+	XII	XV	II

[a] 16B, patient with CG; 1G-4C, patients with PUD. a1–a3, isolates from anthrum biopsies, c1–c3, isolates from corpus biopsies, u: ulcer edge biopsies.
[b] Empty: empty-site PCR-positive amplification of the genome regions flanking the left and right end of *cag*PAI (absence of the entire *cag*PAI).
[c] Empty: *babA*, *babB* or *babC* were not identified in locus C.
[d] R: number of CT repeat at the 5′region of the gene.

interplay, the selection of a specific variant among those that arose during persistent colonisation may occur. In this study, the *cag*PAI with the seven selected markers, *oipA* "on", *vacA* s1-m1 variant, was significantly found in patients with PUD without intra-host variation, perhaps as the result of the selection during disease development. The increasing evidences of the multifactorial aetiology of *H. pylori* related diseases (Akhter et al., 2007; Dorer et al., 2009; Zabaleta, 2012), and the finding of a less extent of microevolution in patients with PUD with the selection of a variant with a specific genotype and phenotype by the intragastric environment may be a reflection rather than a cause of disease.

BabA metastability results also in variants having high potential for periodic activation and deactivation of mucosal binding, appropriate for the intensity of the host response to infection (Bäckström et al., 2004). Homologous recombination of *bab* genes that could control *babA* expression has already been observed in isolates from a single host (Colbeck et al., 2006; Matteo et al., 2011). In this study, a higher level of intra-host variation in *bab* genes was also observed in patients with CG, with low presence of *babA*. Nonetheless, half of the *babA* positive isolates in CG patients exhibited Leb binding "in vitro". On the other hand, patients with PUD showed lower *bab* gene intra-host variations as well as the presence *babA* in isolates from all or several niches that did not mediate Leb binding "in vitro". At this point, Fujimoto et al. (2007) identified a small class of isolates that produced low levels of *BabA* protein and lacked the Leb binding activity. These isolates, classified as functionally *BabA* negative, were found to be most associated with duodenal ulcer, gastric cancer, increased mucosal inflammation and atrophy. In contrast, *BabA* higher producers showing "in vitro". Leb binding activity were most related to chronic gastritis. Skoog et al. (2012) demonstrated that gastric mucin environment can also co-regulate the expression of *H. pylori* genes in relation to host persistence or pathogenesis, like *babA*. Consequently, isolates lacking the Leb binding activity could represent an adaptation of *H. pylori* that enhances survival to mucin glycosylation changes during the disease. The increased intra-host variation found in isolates from CG patients suggests the emergence of variants that regulate the level of *babA* expression ensuring persistence, while the low variation in PUD isolates could reflect the selection of the particular variant with a non-functional *BabA*.

Up to 50% of the strain-specific genes are located in the PZ of the *H. pylori* genome (Romo-González et al., 2009), and the presence of many of them have been postulated as markers of disease outcome with controversial results (Occhialini et al., 2000; de Jonge et al., 2004; Sugimoto et al., 2012). At present, it is assumed that PZ genes are contained in conjugative transposons or their remnants (Kersulyte et al., 2009; Fischer et al., 2010; Farnbacher et al., 2010). Segments of PZs can be lost by both spontaneous and transposable element-mediated deletion processes, much as has been seen with normal chromosomal genes (Kersulyte et al., 2009). Recently, Sugimoto et al. (2012) reported that *jhp*0945, *jhp*0947, and *jhp*0949 genes are significantly more prevalent in Western than in East Asian isolates. In addition, these authors indicated that in Western isolates, the presence of *jhp*0945 was significantly associated with peptic ulcers and gastric cancer, while *jhp*0940 positive isolates were significantly related with absence of peptic ulcers. In our isolates, the prevalence of *jhp*0945, *jhp*0947, and *jhp*0949 genes was similar to that found by Sugimoto et al. (2012). However, *jhp*0945, *jhp*0947, and *jhp*0949 prevailed in isolates recovered from patients with CG, and *jhp*0940 was similarly distributed among the group of the patients studied. Considering the inter-host variation of PZ genes, a similar rate was observed among patients with CG and PUD in almost the five genes studied. This intra-host variation could explain the controversial association between the presence and absence of these PZ genes and disease progression.

Different types of PZs have been described, and *jhp*0926, *jhp*0945, *jhp*0947 homologs are located in PZ type 1, while *jhp*0940 in PZ type 2 (Kersulyte et al., 2009). Isolates with mosaic patterns of both PZ types or remnants of them, as well as isolates harbouring more than one PZ type in different chromosomal loci have been described (Kersulyte et al., 2009; Fischer et al., 2010). The basis for finding well conserved, apparently stable PZ elements in some isolates and only remnants in others, as well as the mechanisms underlying these differences is yet unknown (Kersulyte et al., 2009). However, the similar rate of variation found between CG and PUD isolates might denote that PZ genes evolve under a different pressure caused by the other markers studied. The *vacA* allele inter-host variation was the lowest among all the selected markers used to investigate microevolution. This fact denotes that while some variations can be generated in the absence of mixed colonisation (e.g. slipped-strand mispairing or intrachromosomal recombination), the import of DNA fragments from an exogenous *H. pylori* strain appears to be the principal source of the *vac*A allelic variation (Atherton and Blaser, 2009). Therefore, the intra-host variation of *vacA* alleles suggests the presence of different strains either at another time of colonisation or another unsampled niche of the gastric mucosa. In these cases, the way to define a mixed infection must be considered. In the present study, we used RFLP of two housekeeping genes for strain delineation, hence isolates showing the same banding pattern have the same ancestral origin, and genomic changes in the selected markers to investigate microevolution represent variants of this strain. The RAPD-PCR and AFLP (Amplified Fragment Length Polymorphism)-PCR included the analysis of variations in the whole genome and are very suitable to define mixed infections as well as microevolution in a single host "per se" (Kuipers et al., 2000; Carroll et al., 2004). In this regard, AFLP is a more sensitive fingerprinting technique than RAPD to demonstrate that genetically-related isolates undergone minor genomic variations in a single host (Carroll et al., 2004). In both techniques, additional anlyses are also needed to investigate the cause of the genetic variation. Recently, Patra et al. (2012) denoted that 30 patients were infected with more than one strain and sometimes with strains with 5–6 types of genetic variants, using RAPD-PCR along with the genotyping of several markers like *cagA*, *cag*PAI empty site, *vac*A, *ice*A, *vap*D and IS605 analysis, among others. The authors found that one patient was infected by at least 3 types of "strains" defined as: (i) *cag*A positive/3′ end repeat region of *cagA* Type A/*vac*A s1m1, (ii) *cagA* positive/3′ end repeat region of *cagA* Type B/D/*vac*A s1m1, and (iii) *cagA* negative/*vac*A s2m2. The first two isolates showed a very similar RAPD profile and the last isolate exhibited a distinguishable RAPD banding pattern. The sequence analysis of two housekeeping genes, *ure*B and *rec*A revealed 100% homology in all isolates. The authors concluded that the coexistence of variants of the same strain with different *cagA* and *vac*A genotypes may reflect the physiological differences among the gastric regions of a given host. The prevalence of mixed colonisation/infection would be related with the prevalence of *H. pylori* in each human community. Coinfections with different strains are essential for the panmictic exchange of genes and alleles that favour *H. pylori* persistence. However, coinfection may be transient, or may represent a minor population acting as potential reservoirs of genetic elements for the fittest dominant strain for each host (Atherton and Blaser, 2009). Two or more isolates recovered from a single host that share an ancestral relationship with the fittest strain but have undergone independent genomic alterations (variants), represent the phenomenon termed "microevolution" by Marshall (1997). Therefore, microevolution of *H. pylori* isolates within the host is not a novel finding and according to Argent et al. (2008), it is likely to be a vast underestimation of the true frequency, because the sampling represents a snapshot in time and also involves the tiny areas of the stomach contained in the biopsy specimens. Of note, the significant highest frequency of microevolution found in *cag*PAI, *vacA*, *oipA* and *bab* genes in patients with CG versus patients with PUD under identical methodology is novel, and it could have important biological and clinical implications. However, these results did not exclude microevolution in patients with PUD. In fact, Alvi et al. (2007) described microevolution in *cag*PAI and the *tfs*3 secretion system in isolates obtained sequentially from one patient with duodenal ulcer disease. On the other hand, Avasthi et al. (2011) demonstrated that two isolates recovered after one decade of the original isolation of *H. pylori* from one patient with duodenal ulcer disease were almost identical to the earlier isolate by comparative genomic analysis.

5. Conclusion

The lowest rate of intra-host variation found in the *cag*PAI, *oipA*, *vacA*, and *bab* genes status in patients with PUD may involve the selection of a particular variant along the interplay between bacteria and host during ulceration development. Furthermore, the association of a specific *cag*PAI, *oipA*, *vacA* and *bab* status during the selection supports that these factors interact with each other in the disease development. However, the presence of a particular variant with a specific *cag*PAI, *oipA*, *vacA* and *bab* phenotype may be a refletion of the bacteria-host interplay rather than the cause of the disease, since the same variant was also found in patients with CG. Therefore, the isolation of given a *H. pylori* strain with a particular *cag*PAI, *oip*A, *vacA* and *bab* genes status from colonised patient does not predict disease outcome because their status are not a fixed characteristic.

In regard to the Plasticity Zone genes, the similar rate of variation found among isolates from patients with CG and patients with PUD, might denote that this genomic region evolves under to diverse pressure(s) or additional pressure(s) compared with that the other markers selected to investigate microevolution.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

RA and GZP carried out *Helicobacter pylori* isolation and DNA extraction. RA, MM and CG performed molecular genotyping, sequence analysis, and Leb binding assay. AW, JF and LV collected and selected biopsy specimens. MC undertook the design of the investigation, data interpretation and drafting of the manuscript. All authors have read and approved the manuscript.

Acknowledgment

This study was supported by grants BID 1728 OC-AR PICT 2006-0458 provided by Agencia Nacional de Promoción Científica y Tecnológica and by M064 from Universidad de Buenos Aires (UBACyT 2010-2012).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.meegid.2013.02.024.

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