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Helicobacter pylori heterogeneity in patients with gastritis and peptic ulcer disease

Rita Inés Armitano [a], Mario José Matteo [a], Cinthia Goldman [b], Andrés Wonaga [c,d], Luis Alberto Viola [c], Gerardo Zerbetto De Palma [a], Mariana Catalano [a,*]

[a] *Instituto de Microbiología y Parasitología Médica (IMPAM, UBA-CONICET), Facultad de Medicina, Universidad de Buenos Aires-Consejo Nacional de Investigaciones Científicas y Tecnológicas, Argentina*
[b] *Departamento de Física, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Argentina*
[c] *Centro Integral de Gastroenterología, Buenos Aires, Argentina*
[d] *Servicio de Gastroenterología, Clínica Bazterrica, Buenos Aires, Argentina*

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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

* Corresponding author. Address: Instituto de Microbiología y Parasitología Médica (IMPAM, UBA-CONICET), Facultad de Medicina, Universidad de Buenos Aires, Paraguay 2155, Piso 12, C1121ABG Buenos Aires, Argentina. Tel.: +54 11 5950 9500x2181; fax: +54 11 4964 2554.
E-mail address: catalano@fmed.uba.ar (M. Catalano).

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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)