Pathogenicity island \textit{cag}, \textit{vacA} and IS605 genotypes in Mexican strains of \textit{Helicobacter pylori} associated with peptic ulcers

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Abstract

\textbf{Background:} \textit{Helicobacter pylori} is associated with chronic gastritis, peptic ulcers, and gastric cancer. Two major virulence factors of \textit{H. pylori} have been described: the pathogenicity island \textit{cag} (\textit{cag} PAI) and the vacuolating cytotoxin gene (\textit{vacA}). Virtually all strains have a copy of \textit{vacA}, but its genotype varies. The \textit{cag} PAI is a region of 32 genes in which the insertion of IS605 elements in its middle region has been associated with partial or total deletions of it that have generated strains with varying virulence. Accordingly, the aim of this work was to determine the \textit{cag} PAI integrity, \textit{vacA} genotype and IS605 status in groups of isolates from Mexican patients with non-peptic ulcers (NPU), non-bleeding peptic ulcers (NBPU), and bleeding peptic ulcers (BPU).

\textbf{Methods:} The \textit{cag} PAI integrity was performed by detection of eleven targeted genes along this locus using dot blot hybridization and PCR assays. The \textit{vacA} allelic, \textit{cag} PAI genotype 1 and IS605 status were determined by PCR analysis.

\textbf{Results:} Groups of 16-17 isolates (n = 50) from two patients with NPU, NBPU, and BPU, respectively, were studied. 90% (45/50) of the isolates harbored a complete \textit{cag} PAI. Three BPU isolates lacked the \textit{cag} PAI, and two of the NBPU had an incomplete \textit{cag} PAI: the first isolate was negative for three of its genes, including deletion of the \textit{cagA} gene, whereas the second did not have the \textit{cagM} gene. Most of the strains (76%) had the \textit{vacA} s1b/m1 genotype; meanwhile the IS605 was not present within the \textit{cag} PAI of any strain but was detected elsewhere in the genome of 8% (4/50).

\textbf{Conclusion:} The patients had highly virulent strains since the most of them possessed a complete \textit{cag} PAI and had a \textit{vacA} s1b/m1 genotype. All the isolates presented the \textit{cag} PAI without any IS605 insertion (genotype 1). Combined \textit{vacA} genotypes showed that 1 NPU, 2 NBPU, and 1 BPU patients (66.6%) had a mixed infection; coexistence of \textit{H. pylori} strains with different \textit{cag} PAI status was observed in 1 NBPU and 2 BPU (50%) of the patients, but only two of these patients (NBPU and BPU) had different \textit{vacA} genotypes.

\textbf{Keywords:} \textit{Helicobacter pylori} \textit{cag} PAI, \textit{vacA}, peptic ulcers, Mexico

Introduction

\textit{H. pylori} is a well-recognized pathogen that chronically infects the stomach of up to 50% of the world’s human population. The prevalence of \textit{H. pylori} is high in developing countries; in Mexico its seroprevalence is 66% of the general population and is common in asymptomatic population [1-5].

There are two genotypic characteristics of virulent \textit{H. pylori} strains: the \textit{vacA} gene, and the \textit{cag} PAI region. Virtually all \textit{H. pylori} strains have a copy of \textit{vacA}, but the structure among alleles varies in three regions: the signal (s) region that is present as type s1 (subtype a, b and c) or type s2, the intermediate (i) region that exists in subtype 1 and 2, and the middle (m) region that exists in three different allelic forms (m1, m2a, and
The final structure of the vacA gene is an extensive allelic diversity where all combinations of all those regions have been reported. The s1/m1 genotype encodes a VacA protein, which possesses high vacuolating cytotoxin activity and is associated with severe gastroduodenal diseases [6-9]. Infection by multiple H. pylori strains, defined by different vacA genotypes, is common in Mexico. Nevertheless, the most prevalent vacA genotypes in the Mexican population are s1(b)/m1 and s2/m2, which have been associated with the development of chronic gastritis (CG), peptic ulcers (PU), and gastric cancer (GC) [10-17].

cag PAI encodes components of a type IV secretion system (T4SS), which is used for the translocation of CagA into the gastric epithelium cells, where it is phosphorylated. Phosphorylated CagA interferes with various physiological transduction signals in the host cell and causes pathological cellular responses that contribute to the development of local inflammation and the damage of the gastric mucosa. The elimination of T4SS function significantly reduces the accumulation of phosphorylated CagA in the host cell and the secretion of interleukin 8 (IL-8) [18]. The presence of cag PAI has been associated with the development of CG, PU, and GC [19].

In Mexico, studies of cag PAI and its relationship with the infection outcomes have focused on the cagA gene and its product, protein CagA, which have been used as biomarkers of this genetic region. These studies have shown a correlation between serologic detection of antibodies against CagA and the identification of the infecting H. pylori cagA+ strains [2-4,12,20,21]. A series of studies have shown a close association between CagA antibodies and the development of gastroduodenal pathologies such as PU and GC [12,22,23]. The presence of cagA as a biomarker for cag PAI and gastric colonization with cagA+ strains associated with gastrointestinal diseases has been documented by PCR in isolated bacterial DNA and has also been detected by hybridization in situ from biopsies or by microarray hybridization [4,5,10-12,14-16,24,25].

Nevertheless, many studies have demonstrated that T4SS integrity is needed for translocation of CagA protein into gastric epithelium cells and that the insertion of IS605 elements between cag I and cag II of cag PAI has been implicated in partial or total deletions that have generated H. pylori strains with varying virulence [26,27].

In Mexico, three studies have addressed the correlation between genetic integrity in cag PAI in multiple isolates obtained from individual patients with gastroduodenal diseases. One study characterized the vacA genotypes and evaluated the presence of cag PAI by PCR amplification of the cagA in four children with chronic abdominal pain (CAP), eight adult patients with PU and one with non-ulcer dyspepsia (NUD). This study also characterized the diversity of cag PAI by microarray genotyping in 51 isolates from three of the adult with PU and four of the pediatric patients [16]. The second study showed homogeneous cag PAI+ content in eight adults and ten pediatric patients with CAP, PU or NUD, after evaluating the presence of cagA, cagE, cagT and cag10 genes by PCR [25]. The third study, which used DNA microarrays, showed that the presence of a complete cag PAI was significantly more frequent for isolates from patients with GC than for isolates from non-atrophic gastritis (NAG) and duodenal ulcer (DU) patients [5]. Nevertheless, the integrity of cag PAI and vacA genotypes as important contributors to the risk of PU development, and its most common complication, BPU, had not previously been studied in the Mexican population. Accordingly, the goal of this work was to establish the cag PAI, vacA and IS605 status in groups of single-colony isolates from Mexican patients with non-peptic ulcers and (bleeding or not bleeding) peptic ulcers.

Methods
Study subjects
Two patients with gastric or duodenal ulcers with ongoing or recent (24 h) bleeding (BPU) were recruited and the gastric biopsy samples from each were taken into the first 24 hours. Four additional patients were included in the study as controls: two patients with NBPU and two on whom an endoscopy was performed because of non-ulcer dyspepsia and/or gastro-esophageal reflux (NPU). None of the patients had clinical history of mucosa associated lymphoid tissue (MALT) lymphoma or Crohn’s disease. 50 clinical H. pylori isolates were obtained from biopsy specimens: 17 (8 and 9) from the two patients with BPU; 17 (12 and 5) from the two patients with NBPU, and 16 (9 and 7) from the patients with NPU. All six patients were recruited from a Mestizo-Mexican population in a previous cross-sectional, prospective, observer-blind study carried out between November 1995 and August 1997 [4]. The subjects ranged from 53 to 72 years old (mean 61 years), and the F/M sex ratio was 1:1 in the NBPU and NPU groups and 0:2 in the BPU group. At least seven biopsies from the antral and corpus gastric mucosa were taken during the same endoscopic sessions of each patient.

H. pylori culture and chromosomal DNA extraction
H. pylori was cultured by smearing biopsy specimens on the surfaces of horse blood agar plates. The colonies obtained were identified as H. pylori according to standard criteria, including typical cell morphology, negative Gram staining, and biochemical testing. The rest of the colonies from each plate were harvested in batches to
obtain a new culture on blood agar plates. Between 9 and 12 colonies were picked separately from these plates and were then passed onto individual plates. Each of these plates was propagated three more times for the obtaining of biomasses and extraction of chromosomal DNAs as previously reported [10]. In the current study these chromosomal DNAs were used to carry out all the genotyping assays after their characterization by PCR amplification of the *H. pylori* 16S rRNA gene, using the HP16-219 and HPGR-16S pair of primers (see Table 1).

**PCR Primer Design**

Using the genome sequence of the *H. pylori* 26695 strain as reference (ATCC 700392) [GenBank: AE000511.1] [28], pairs of primers were designed to carry out specific PCR amplifications for one or two of selected genes from the region cag I: cag6, cag6-7, cag10, cag13 and cag16, cagT and cagS; and from the cag II region of cag PAI: cagQ, cagM, cagI, cagE and cagA [26,27]. The cagS-Q-F and cagS-Q-R pair of primers was designed to amplify the DNA cagS-Q fragment and to detect the presence of the IS605 between the cag I and cag II regions of cag PAI. The TnpB-cagSF and TnpB-cagSR primers were designed to detect the IS605 contiguous to the cag II region that characterizes the genotype 2 of cag PAI previously reported [26]. To determine if IS605 was present elsewhere in the genome, the primers IS605F and IS605R were used. For amplification of cagA, the primers

| Primer | Sequence (5'-3') | Size of amplified fragment | Annealing temperature °C | Position in genome of reference (H. pylori 26695)* |
|--------|-----------------|---------------------------|--------------------------|-----------------------------------------------|
| 16S rRNA |                |                           |                          |                                               |
| HPGF-16S | CAATCAGCGTCAGTAATGTTC | 516                        | 55                       | 1208359-1208379                               |
| HPGR-16S | CTAAGAGATCAGCCTATGTC | 1208855-1208875            |                          |                                               |
| cag PAI genes |  |                           |                          |                                               |
| cag1-F | TTCATTAGGTCTCATTGGAGCAG | 230                        | 62                       | 547429-547452                                 |
| cag1-R | TTGGCTTACGTGTTTTGTTGG  | 547639-547659              |                          |                                               |
| cag1-F | TTCATTAGGTCTCATTGGAGCAG | 430                        | 66                       | 547429-547452                                 |
| cag1-2-R | CCGTTACGGCGGCTTTATTAC | 645                        | 66                       | 551152-551175                                 |
| cag5-F | GGCCTACTTATCTCTTCACCTC | 587                        | 55                       | 551776-551797                                 |
| cag5-R | GGGGATTTTATCGCTTACCG   | 598                        | 66                       | 554726-554747                                 |
| cag7-F | AAACCGGTCATCAATGCTTGG  | 986                        | 66                       | 555692-555712                                 |
| cag7-R | AGAAAAAGGTGCTTGGGATTTG | 627                        | 62                       | 562353-562372                                 |
| cag9-F | ATGTCGCCCAACACAATGCC  | 645                        | 66                       | 562959-562980                                 |
| cag9-R | CCATTACGCACAAAGTTTACCG | 587                        | 55                       | 565331-565355                                 |
| cagT-F | GTCACCATCTCATAAAGGATTACGC | 555                        | 66                       | 565863-565886                                 |
| cagT-R | GATTTCGCAAGTATTCATTCTCTTCT | 342                        | 54                       | 566203-566222                                 |
| cagS-F | CAATGGTTTTTATGATTACCCG | 342                        | 54                       | 566527-566545                                 |
| cagS-R | AAGGGAGCGTTAGATAAAGG  | 342                        | 54                       | 566527-566545                                 |
| cagS-Q-F | TCTAACGCTCCCTGTGTTGATGCG | 687                        | 66                       | 566532-566555                                 |
| cagS-Q-R | TTGGTGTGTAATGGTTTTGAGCCG | 687                        | 66                       | 567196-567219                                 |
| cagQ-F | CCAAAAACATTAACACAAACACG | 292                        | 66                       | 567200-567222                                 |
| cagQ-R | CCGAACAAAGGAACTCAACACAC     | 627                        | 62                       | 567468-567492                                 |
| cagM-F | GGTGCGTGGGAGTGGTTTTCG   | 485                        | 66                       | 568752-568773                                 |
| cagM-R | TGACCTTTATCTATTGTTGTACGGG | 485                        | 66                       | 569213-569237                                 |
| cagI-F | TGTCCTTCCTCAAAAGGTCGCG  | 510                        | 62                       | 571644-571663                                 |
| cagI-R | CTCTCGAAGCGCTAATACATCG   | 510                        | 62                       | 572131-572154                                 |
| cagE-F | AGTATGCGTTGTTAGGCGAAGTC  | 923                        | 64                       | 575506-575528                                 |
| cagE-R | TGGGCGAAATGCGAATGCGAG   | 923                        | 64                       | 576409-576429                                 |
| caga-F | GCCTATATTTAATATCCTGCTATCAC | 744                        | 55                       | 581555-581581                                 |
| caga-R | ATGAGATTGTTGCACTATTTACCG | 744                        | 55                       | 582277-582299                                 |
| IS605-F | TTTGATTTAAACGGATGTTGG  | 1021                       | 55                       | 13986-14007 (AC000108)*#                       |
| IS605-R | TGTTTTTGAATAGGGGATTGGGAT | 1021                       | 55                       | 14983-15002                                    |
| TnpB-cag5-F | CGTCCTAAGCCCATGTTCAAAACC | 1597                       | 56                       | 14038-14061 (AC000108)*#                      |
| TnpB-cag5-R | TCTACTGCCTCCCCTTTTGATGTC | 1597                       | 56                       | 15612-15635                                   |

*GenBank: AE000511.1; #GenBank: NCTC11638.*
cagA-F and cagA-R were designed from a consensus sequence of a set of 26 cagA gene-sequences reported [GenBank: AB015404-16; AF202972-3; AB011791-2; AF249275; AF001357; L11714; AF083352; AF282853; AF247651; AF001483 and AF003397], using the MacVector 2.1 molecular biology program. Table 1 shows the nucleotide sequence of PCR primers used for the generation of all these DNA-fragments. The parameters for the amplification of IS605 in the genome were 94°C for 45 seconds, 54°C for 1 minute and 30 seconds, and 72°C for 1 minute and 30 seconds, for a total of 30 cycles. The amplification parameters for DNA fragment cagS-Q were 94°C for 1 minute, 50°C for 1 minute, 72°C for 2 minutes, for a total of 30 cycles.

Cloning of the cag PAI genes

Eleven DNA fragments corresponding to individual selected cag PAI genes and two DNA fragments that included the cag6-7 and cagS-cagQ genes were generated by PCR. The thermal cycling conditions were at 94°C for 30 seconds, the primer annealing temperature ranged from 54-66°C (depending on the melting temperature of each set of primers that were used; see Table 1) for 30 seconds, and 72°C for 30 seconds, for a total of 30 cycles. The PCR products were cloned directly into the linearized pCR®2.1-TOPO® vector to obtain a genetic library of cag PAI formed by thirteen plasmids. The thirteen cloned fragments showed 100% identity in their nucleotide sequence when they were compared with the reported sequence of the H. pylori strain 26695, using the BLAST program.

Southern blot hybridization

For all the H. pylori strains and controls, 500 μg of genomic DNA were transferred to a nylon membrane (Hybond-N+) with sodium hydroxide. The genomic DNAs were hybridized with each one of the plasmids of the genetic library of cag PAI, which were previously digested with the EcoRI overnight at 37°C. The plasmid DNAs were labelled with thermostable alkaline phosphatase, Alkphos Direct Labelling and Detection System and CDP-Star (Amersham Pharmacia Biotech). The membrane was pre-hybridized for 2 hours at 55°C, and hybridized at 55°C overnight. The membrane was washed three times at 58°C, in a shaking water bath for 15 minutes, transferred to a clean container and washed twice with secondary wash solution for 10 minutes at room temperature. Excess of wash solution was removed from the membrane and the CDP-Star substrate was added and incubated at room temperature for 5 minutes. Afterwards the membrane was wrapped in plastic film and autoradiography was performed with a sheet of Hyperfilm™ ECL (Amersham Pharmacia Biotech) at different lengths of time. The membranes were stripped from the previously hybridized probe and stored in hybridization bags at 4°C to be used with another probe.

vacA genotyping by specific PCR amplification

The vacA signal and middle regions were typed by using the primers as described previously by Atherton et al. [6]. The isolates were at first identified as type s1 or s2 and type m1 or m2. All strains with signal region type s1 were further characterized into s1a or s1b variants by performing three separate PCR assays. H. pylori 26695 (cag PAI+, vacA s1a/m1), J99 (ATCC 700824) (cag PAI+, vacA s1b/m1), Tx30a (ATCC 51932) (cag PAI-, vacA s2/m2), and 86-313 (cag PAI-, vacA s2/m2) strains were used as controls for vacA allele detection [28-30]. Distilled water was used as an internal-reaction negative control. Thermal cycling conditions for each set of primers (0.5 μM) were at 95°C for 1 minute, 52°C for 1 minute, for a total of 35 cycles.

Results

We investigated the integrity of cag PAI, vacA alleles, and the presence of IS605 in groups of single-colony isolates from three pairs of Mexican patients with NPU, NBPU, and BPU. To analyze the integrity of the cag PAI region of groups of 5-12 single-colonies (n = 50), Southern blot hybridizations were performed. The analysis was based on the detection of eleven targeted genes homogeneously distributed along this locus using the cag PAI genetic library as a probe. An example of detection of the cagA gene by hybridization is illustrated in Figure 1. To confirm the result of the hybridizations, the genomic DNAs were used for detection of cagA gene by PCR using the pair of consensus primers described in Table 1. The percentage of detection of cagA gene by PCR, with respect to those identified by hybridization, was 97.7%. Thus, a gene marker was defined as positive if it was detected by dot blot and not by PCR. The results showed that 90% (45/50) of the isolates were positive for all of the studied genes of cag PAI, whereas 4% (2/50) and 6% (3/50) had an incomplete cag PAI or were negative for all the analyzed genes, respectively. Co-existence of strains with different cag PAI status was observed in 50% (3/6) of the patients (see Table 2). 2 BPU patients were infected with multiple strains, some of which had a complete cag PAI, and others that didn’t: 1/8 (12.5%) and 2/9 (25%), respectively. Meanwhile, 1 NBPU patient was infected with strains with a complete cag PAI but also with strains with an incomplete island 2/12 (16%). One of these strains showed deletions of the cagW, cagT, cagI, including deletion of the cagA gene, while the other did not have the cagM gene. Thus, the presence of the complete cag PAI was found in 100% (16/16); 88.2% (15/17);
82.3% (14/17) of the clinical isolates from the group of patients with NPU, NBPU, and BPU, respectively. The patients with NPU, NBPU, and BPU, respectively. The results of the Southern hybridization were confirmed by PCR amplifications of the eleven cag PAI gene markers in the five strains that had the incomplete or lacked the cag PAI. The PCR amplifications were similar to those obtained by the hybridization method.

PCR analysis was performed in order to determine whether the clinical isolates had a pathogenicity island that was uninterrupted or one that was divided in two regions as the result of insertion of a single-copy of IS605 element, similar to genotypes 3 described previously [26]. These PCR assays were carried out using specific primers for sites upstream and downstream of this insertion (cagS-Q-F and cagS-Q-R; Table 1) to amplify the cagS-Q DNA fragment. DNA fragments of 2,567 pb or 687 pb were predicted whether or not the cag PAI was split by an IS605 element. The results of this analysis showed the presence of a cagS-Q fragment of 687 pb in 100% (16/16) of the isolates of the patients with NPU. In the group of patients with BPU, 70.5% (12/17) of the isolates resulted positive for this DNA fragment. Three fifths (3/5) of isolates that were negative for this DNA fragment were also negative for the cag PAI by dot blot hybridization (11.7%) (cag PAI- isolates; see Table 2). The 2/5 remaining BPU isolates were negative for the cagS-Q DNA fragment, even though they were positive for cagS and cagQ genes by dot blot hybridization. Similarly, one isolate from a NBPU patient (1/9) was negative for this fragment but positive for cagS and cagQ genes by Southern blot. PCR amplifications were performed in order to rule out the possibility that these three strains may have had an IS605-based rearrangement in the island similar to genotype 2 in the NCTC 11608 strain (where the cag I and cag II regions are separated by a large fragment of chromosomal DNA flanked by two IS605 sequences) [26,27]. The tnPB-cagS-F and tnPB-cagS-R primers were used to amplify a 1,597 pb DNA fragment between the cagS gene and the tnPB transposases of the adjacent IS605,

| cagPAI | IS605 present outside cag PAI | vacA genotype |
|--------|-------------------------------|--------------|
|        | Non-peptic ulcers | Non-Bleeding Peptic ulcers | Bleeding peptic ulcers | Total % of isolates |
| CP + | Patient 1 | Patient 2 | Patient 1 | Patient 2 | Patient 1 | Patient 2 | Patient 1 | Patient 2 |
|       | s1b/m1 (9)* | s1b/m2 (5), s0/ m1 (1) | s1b/m1 (7) s2/ m2 (1) | s1b/m1 (3), s1b/m2 (1), s0/m1 (1) | s1b/m1 (7) | s1b/m1 (6), s1b/m0 (1) |
| CP + | + | s1b/m2 (1) | s1b/m1 (2) | s1b/m1 (1) | s1b/m1 (1) | 84 |
| CP - | - | s0/m1 (1) | s1b/m1 (1) | 6 |
| CP +/- | + | s1b/m1 (1) | s1b/m1 (2) | 6 |
| CP +/- | - | s1b/m1 (1) | s1b/m1 (2) | 2 |

(*) numbers of isolates; CP+, complete; CP-, without; CP+/-, incomplete; s0, untypeable for signal region; m0, untypeable for middle region. Patient No 1: Mestizo female, 54 years old, without peptic ulcers or ulcer history; patient No 2: Mestizo male, 53 years old, without peptic ulcers or ulcer history; patient No 3: Mestizo male, 63 years old, with ulcer history, non-bleeding duodenal ulcers; patient No 4: Mestizo female, 65 years old, with ulcer history, non-bleeding duodenal ulcers; patient No 5: Mestizo male, 61 years old, without ulcer history, bleeding duodenal ulcers; patient No 6: Mestizo male, 72 years old, with ulcer history, bleeding gastric ulcers.
using the NCTC 11608 strain as a positive control. The IS605 insertion sequence was not found, and it can therefore be assumed that the cag PAI was presented as an uninterrupted unit in 94% (47/50) of the analyzed isolates (cag PAI 1 genotype) [26].

In this study, 2 strains (4%) presented partial deletions of cag PAI. The presence of IS605 anywhere in the H. pylori chromosome is thought to be involved in cag deletions [26]. To determine whether or not the clinical isolates had the IS605 insertion elsewhere in the chromosome, the IS605-F and IS605-R primers were used to amplify a tnpA-tnpB DNA fragment of 1,016 pb. The results of this analysis showed that the IS605 insertion was present in 8% (4/50) of the isolates. One isolate with a complete cag PAI was present in a patient with NPU, and three isolates were present in only one patient with NBPU: two of the isolates had a complete cag PAI and another had an incomplete cag PAI. Thus, the complete cag PAI/IS605-genotype of the strains was predominant in the different patients in this study.

To account for the possibility that the different patients might have been infected with multiple strains of H. pylori, we investigated the vacA genotypes of the clinical isolates. Three combinations of vacA s and m regions were identified: 38 (76%) of the strains had the s1b/m1 genotype that was previously described as the most prevalent vacA genotype in Mexican populations [10,14]. The second most frequent vacA genotype was s1b/m2, which was present in 7 (14%) strains, whereas the s2/m2 genotype, previously reported as the most prevalent genotype in PU patients from north-eastern of Mexico [11,12], was only present in 1 (2%) of NBPU isolates. The s2/m1 genotype described previously was not identified [10-12,15]. Untypeable vacA genotypes have been previously described in Mexican populations. In this study we found 3 (6%) strains where vacA region m1 was amplified, but vacA s region was not detected (1 NPU, and 2 NBPU isolates). Likewise, 1 BPU isolate (2%) was s1b/m0, which accounts for the possibility that an additional subfamily of s and m genotypes might exist in Mexican isolates, given that in this study as in those reported elsewhere in México, the s1c and m3 vacA alleles, which have been described in some populations [31], were not investigated [10,11,14,17]. In this study, evidence of infection by more than one H. pylori strain was found in 66.6% (4/6) of the patients, where at least two vacA allotypes were detected. This confirms the multi-strain nature of the H. pylori infection previously described in the Mexican population [10,12-14] (see Table 2).

**Discussion**

In this study which used eleven selected cag PAI genes as a probe to carry out the characterization of cag PAI by Southern blot hybridization, we found that among the 50 H. pylori isolates, a completed cag PAI was detected in 45 (90%) of them; 2 (4%) presented an incomplete cag PAI, whereas in 3 (6%) the cag PAI was not detected. Thus, most of our isolates possessed a complete cag PAI. Three previous Mexican studies are consistent with our results. The first of these, which evaluated the presence of four cag PAI genes markers by PCR assays, showed that 78.6% of the studied isolates had a complete cag PAI, while 15.1% did not have it, and 6.2% had partial deletions [25]. The second study, which used hybridization microarrays to characterize this genetic locus, showed that 18/29 (62%) of the studied isolates had a complete cag PAI [5]. Nevertheless, these two studies and those previously published about the Mexican population did not carry out the correlation between the integrity of cag PAI and vacA genotypes. Our data showed that 90% of the strains had a complete cag PAI and that the major vacA s1b/m1 and s1b/m2 genotypes were present in 34/50 (68%) and 7/50 (14%) of the strains, respectively, whereas minor s2/m2, s1b/m0 and s0/m1 genotypes were present in 1 (2%), 1 (2%) and 2 (4%). In 2/50 (4%) of the isolates with an incomplete cag PAI, the s1b/m1 and s0/m1 genotypes were present. Meanwhile, the vacA genotype was s1b/m1 in the 3/50 (6%) of the strains with an undetected cag PAI. Thus, strains with a complete cag PAI and vacA s1b/m1 genotype were the most common. In one of the NPU patients, the clinical isolates were highly homogenous: 100% (9/9) of the isolates had a complete island with a vacA s1b/m1 genotype (see Table 2). Nevertheless, 4/6 (66.6%) of the studied subjects had multiple genotype infections. The most notorious of them was one of the NBPU patients who not only had three strains with different vacA genotypes, including the most prevalent genotype in north-eastern of Mexico, the s2/m2, and untypeable s0/m1, but also 10/12 strains with a complete cag PAI, and 2/12 incomplete islands (see Table 2). One of these last strains showed deletions of the cagW, cagT, cagI, including deletion of the cagA gene, while the other strain, the cagM gene was deleted. In addition, this NBPU strain represented 1/4 of the strains which had an IS605 insertion somewhere in the chromosome. The deletions in our isolates are quite different from that reported previously [5], which showed that almost 24% of the deletion in cag PAI corresponded to the cag2 gene and has been reported as nonessential for functional activity of theT4SS [18].

Our data showed for first time that the 94% of the Mexican isolates had the cag PAI genotype described previously [26], which did not have an IS605 insertion between cagI and cagII regions; the remaining 6% of the isolates lacked the cag PAI.
Conclusion
The presence of the cag PAI and vacA genotypes in different subjects was analyzed according to disease group: no differences were found between BPU and NBPU isolates, in which the complete cag PAI was present in 88.2% and 82.3%, respectively. The presence of vacA s1b/m1 genotype in NBPU (76%) is different when compared with BPU (94%). Thus, we can conclude that although the integrity of cag PAI is similar in NBUP and BPU isolates, the presence of vacA s1b/m1 genotype in BPU has a slightly higher presence when compared with NBPU isolates.

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Authors’ contributions
GCR, FAR, MLTB carried out the molecular genetic studies, and microbiological procedures. GCR, ECLP, SPDL analyzed and interpreted the data, and help to drafting the manuscript. GRAG, YLP conceived of the study, participated in its design and coordination of the experiments, and drafted the manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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References
1. Torres J, Leal-Herrera Y, Perez-Perez G, Gomez A, Camorlinga-Ponce M, Cedillo-Rivera R, Tapia-Convay R, Muñoz O. A community-based seroepidemiological study of Helicobacter pylori infection in Mexico. J Infect Dis 1998, 178:1089-1094.
2. Torres J, Camorlinga-Ponce M, Perez-Perez G, Muñoz L, Muñoz O. Specific serum immunoglobulin G response to urease and CagA antigens of Helicobacter pylori in infected children and adults in a country with high prevalence of infection. Clin Diag Lab Immunol 2002, 9:97-100.
3. Camorlinga-Ponce M, Flores-Luna L, Lázaro-Ponce E, Herrera R, Bernal-Salahgún F, Abdo-Francis JM, Aguirre-Garcia J, Muñoz N, Torres J. Age and severity of mucosal lesions influence the performance of serologic markers in Helicobacter pylori-associated gastroduodenal pathologies. Cancer Epidemiol Biomarkers Prev 2008, 17:2498-2504.
4. Castillo-Rojas G, Ballesteros MA, Ponce de León S, Morales-Espinosa R, Cravioto A, López-Vidal Y. Bleeding peptic ulcers and presence of Helicobacter pylori by various tests: a case-control study. Eur J Gastroenterol Hepatol 2002, 14:1113-1116.
5. Romero-González G, Salama NR, Burgueño-Ferreira J, Ponce-Castañeda V, Lázaro-Ponce E, Camorlinga-Ponce M, Torres J. Differences in genome content among Helicobacter pylori isolates from patients with gastritis, duodenal ulcer, or gastric cancer reveal novel disease-associated genes. Infect Immun 2009, 77:2201-2211.
6. Atherton JC, Cao P, Peek RM Jr, Tummuru MK, Blaser MJ, Cover TL. Mosaicism in vacuolating cytotoxin alleles of Helicobacter pylori. Association of specific vacA types with cytotoxin production and peptic ulceration. J Biol Chem 1995, 270:17771-17777.
7. van Doom LJ, Figueredo C, Sanna R, Pena S, Middolo P, Ng EK, Atherton JC, Blaser MJ, Quint WG. Expanding allelic diversity of Helicobacter pylori vacA. J Clin Microbiol 1998, 36:2597-2603.
8. Atherton JC, Peek RM Jr, Tham KT, Cover TL, Blaser MJ. Clinical and pathological importance of heterogeneity in vacA, the vacuolating cytotoxin gene of Helicobacter pylori. Gastroenterology 1997, 112:92-99.
9. Rheed JL, Lettley DP, Mohammadi M, Hussein N, Mohagheghi MA, Eshagh Hosseini M, Atherton JC. A new Helicobacter pylori vacuolating cytotoxin determinant, the intermediate region, is associated with gastric cancer. Gastroenterology 2007, 133:926-936.
10. Morales-Espinosa R, Castillo-Rojas G, González-Valencia G, Ponce de León S, Cravioto A, Atherton JC, López-Vidal Y. Colonization of Mexican patients by multiple Helicobacter pylori strains with different vacA and cagA genotypes. J Clin Microbiol 1999, 37:3001-3004.
11. Garza-González E, Bosques-Padilla FJ, Tijerina-Menchaca R, Perez-Perez GI. Characterization of Helicobacter pylori vacA genotypes from the north-eastern region of Mexico. Clin Microbiol Infect 2004, 10:41-45.
12. Garza-González E, Pérez-Pérez GI, Tijerina-Menchaca R, Maldonado-Garza HJ, Bosques-Padilla FJ. Genotypes of Helicobacter pylori y su asociacion con la respuesta inmune del hospedero. Rev Gastroenterol Méx 2002, 67:155-160.
13. González-Valencia G, Atherton JC, Muñoz O, Dehesa M, la Garza AM, Torres J. Helicobacter pylori vacA and cagA genotypes in Mexican adults and children. J Infect Dis 2000, 182:1450-1454.
14. López-Vidal Y, Ponce-de-León S, Castillo-Rojas G, Barreto-Zúñiga R, Torre-Delgadillo A. High diversity of vacA and cagA Helicobacter pylori genotypes in patients with and without gastric cancer. PLoS One 2008, 3:e3849.
15. Paniguia GL, Monroy E, Rodríguez R, Amoniz S, Rodríguez C, Cortés JL, Camacho A, Negrete E, Vaca S. Frequency of vacA, cagA and babB2 virulence markers in Helicobacter pylori strains isolated from Mexican patients with chronic gastritis. Ann Clin Microbiol Antimicrob 2010, 9:14.
16. Salama NR, González-Valencia G, Deheasera B, Aviles-Jimenez F, Atherton JC, Graham DY, Torres J. Genetic analysis of Helicobacter pylori strain populations colonizing the stomach at different times postinfection. J Infect Chemother 2001, 189:3834-3845.
17. Martínez-Camollo DN, Garza-González E, Betancourt-Linares R, Monico-Manzano T, Antunez-Rivera C, Roman-Roman A, Flores-Aliaro E, Illades-Aguir R, Fernandez-Tilapa G. Association of IL1B -511C/-317T haplotype and Helicobacter pylori vacA genotypes with gastric ulcer and chronic gastritis. BMC Gastroenterol 2010, 10:126.
18. Fischer W, Püls J, Buhndorf R, Gebert B, Odenbreit S, Haas R. Systematic mutagenesis of the Helicobacter pylori cag pathogenicity island: essential genes for CagA translocation in host cells and induction of interleukin-8. Mol Microbiol 2001, 42:1337-1348.
19. Backert S, Schwarz T, Miehlke S, Kirsch C, Sommers C, Kucko T, Gerhard M, Goebel UB, Lahn N, Koenig W, Meyer TF. Functional analysis of the cag pathogenicity island in Helicobacter pylori isolates from patients with gastritis, peptic ulcer, and gastric cancer. Infect Immun 2004, 72:1043-1056.
20. López-Camillo L, Torres-López J, Galván-Portillo M, Muñoz L, López-Cervantes M. Helicobacter pylori-CagA serosensitivity and nitrite and ascorbic acid food intake as predictors for gastric cancer. Eur J Cancer 2004, 40:1752-1759.
21. Garza-González E, Bosques-Padilla FJ, Tijerina-Menchaca R, Flores-Gutiérrez JP, Maldonado-Garza HJ, Pérez-Pérez GI. Comparison of endoscopy-based and serum-based methods for the diagnosis of Helicobacter pylori. Can J Gastroenterol 2003, 17:101-106.
22. Torres J, Pérez-Pérez GI, Leal-Herrera Y, Muñoz O. Infection with CagA+ Helicobacter pylori strains as a possible predictor of risk in the development of gastric adenocarcinoma in Mexico. Int J Cancer 1998, 78:299-300.
23. Garza-González E, Bosques-Padilla FJ, Pérez-Pérez GI, Flores-Gutiérrez JP, Tijerina-Menchaca R. Association of gastric cancer, HLA-DQA1, and infection with Helicobacter pylori CagA+ and VacA+ in a Mexican population. J Gastroenterol 2004, 39:1138-1142.
24. Camorlinga-Ponce M, Romo C, González-Valencia G, Muñoz O, Torres J: Topographical localisation of cagA positive and cagA negative Helicobacter pylori strains in the gastric mucosa; an in situ hybridisation study. J Clin Pathol 2004, 57:822-828.

25. Reyes-Leon A, Atherton JC, Argent RH, Puente JL, Torres J: Heterogeneity in the activity of Mexican Helicobacter pylori strains in gastric epithelial cells and its association with diversity in the cagA gene. Infect Immun 2007, 75:3445-3454.

26. Censini S, Lange C, Xiang Z, Crabtree JE, Ghia P, Borodovsky M, Rappuoli R, Covacci A: cag, a pathogenicity island of Helicobacter pylori, encodes type I-specific and disease-associated virulence factors. Proc Natl Acad Sci USA 1996, 93:14648-14653.

27. Akopyants NS, Clifton SW, Kersey JE, Crabtree JE, Youree BE, Reece CA, Bukanov NO, Drazek ES, Roe BA, Berg DE: Analyses of the cag pathogenicity island of Helicobacter pylori. Mol Microbiol 1998, 28:37-53.

28. Alm RA, Ling LS, More DT, King BL, Brown ED, Dog S, Smith DR, Noonan B, Guild BC, Dejonge BL, Carmel G, Tummino PJ, Casulo A, Uria-Nickelsen M, Mills DM, Ives C, Gibson R, Merberg D, Mills SD, Jiang Q, Taylor DE, Voss GF, Trust TJ: Genomic-sequence comparison of two unrelated isolates of the human gastric pathogen Helicobacter pylori. Nature 1999, 397:176-180.

29. Tumuru MK, Cover TL, Blaser MJ: Cloning and expression of a high-molecular-mass major antigen of Helicobacter pylori: evidence of linkage to cytotoxin production. Infect Immun 1993, 61:1759-1809.

30. Rittig MG, Shaw B, Letley DP, Thomas RJ, Argent RH, Atherton JC: Helicobacter pylori-induced homotypic phagosome fusion in human monocytes is independent of the bacterial vacA and cag status. Cell Microbiol 2003, 5:887-899.

31. Han SR, Schenber HJ, Bhakdi S, Loos M, Hanauer MJ: vacA genotypes and genetic diversity in clinical isolates of Helicobacter pylori. Clin Diag Lab Immunol 1998, 5:139-145.

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