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Relationship between *Helicobacter pylori* virulence factors and regulatory cytokines as predictors of clinical outcome

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Abstract

H. pylori infection is highly prevalent in Chile (73%). Usually a minority of infected patients develops complications such as ulcers and gastric cancer that have been associated with the presence of virulence factors (*cagA*, *vacA*) and host T helper response (Th1/Th2). Our aim was to evaluate the relationship between strain virulence and host immune response, using a multiple regression approach for the development of a model based on data collected from *H. pylori* infected patients in Chile. We analyzed levels of selected cytokines determined by ELISA (IL-12, IL-10, IFN-γ and IL-4) and the presence of *cagA* and *vacA* alleles polymorphisms determined by PCR in antral biopsies of 41 patients referred to endoscopy. By multiple regression analysis we established a correlation between bacterial and host factors using clinical outcome (gastritis and duodenal ulcer) as dependent variables. The selected model was described by: clinical outcome = 0.867491 (*cagA*) + 0.0131847 (IL-12/IL-10) + 0.0103503 (IFN-γ/IL-4) and it was able to explain over 90% of clinical outcomes observations (R^2=96.4). This model considers that clinical outcomes are better explained by the interaction of host immune factors and strain virulence as a complex and interdependent mechanism.

Keywords

H. pylori; virulence factors; cytokines; gastroduodenal ulcer

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1. Introduction

Helicobacter pylori, a Gram negative bacterium that colonizes gastric epithelium of the human stomach, has been associated to the development of chronic gastritis. In most patients (80%), *H. pylori* does not cause clinical symptoms and the infection can persist for lifetime without further problems. A minority of the infected patients will develop gastroduodenal disease such as peptic ulcer. Furthermore, a small percentage of infected patients will develop distal gastric cancer, depending on a variety of factors, including strain virulence and host immune responses [1], which is particularly important in Chile, a country with 73% of its population infected [2] and a GC mortality of 19/100.000 habitants [3].

Significant effort has been made to evaluate specific virulence factors present in a subset of strains which has been associated with more severe disease. Strains harboring a functional CagA-PAI and a VacA s1/m1 genotype have been detected at higher frequency in patients with duodenal ulcer, atrophic gastritis and gastric cancer compared with strains lacking this virulence factors [4]. VacA is a 95 kDa secreted cytotoxin which induces apoptosis and is also involved in immunomodulation and colonization [5]. CagA, a 128 kDa immunodominant antigen, encoded by a chromosomal pathogenicity island (Cag-PAI), which also containes genes coding for a type IV secretion system that is able to translocate CagA to the cytoplasm of the host cell, were it is phosphorylated at specific tyrosine residues by a Src Kinases family and activates SHP-2 tyrosine phosphatase which interferes with intracellular signaling leading to morphological as well as functional changes in epithelial cells [4].

H. pylori binds to the gastric epithelial cells through adhesins and persist in its niche for decades [6]. In strains carrying the Cag-PAI type IV secretion apparatus, translocation of CagA into epithelial cells are allowed eliciting the release of Interleukin (IL)-8 and others chemoquines by these cells. Chemoquines lead the recruitment of mononuclear and polymorphonuclear cells (PMNs), initiating the characteristic inflammation of *H. pylori* infected patients [7–8]. In addition, CagA induces disruption of tight junctions facilitating VacA-mediated disruption of epithelial barrier inducing apoptosis. TNF-α and other pro-inflammatory cytokines produced by local macrophages mediate apoptosis and may also lead to disruption of epithelial cells [9].

Although *H. pylori* itself can induce mucosal injury, the local immune response mechanisms have been involved in the pathogenesis of the disease. A large number of studies in mouse and human models have shown that a T lymphocyte helper (Th) type 1 response characterized by an up regulation of IFN-γ is associated with severe pathology outcomes such as ulcers [10]. A protective role has been assigned to the presence of Treg and Th2 type cells that balance some of the effects of Th1 bias. In athymic mice which have been transfuse with CD4+, the absence of CD4+CD25+ cells produces severe inflammation and elevated IFN-γ secretion [11]. In opposition, the presence of Th2 cytokine type IL-4 is associated with lower levels of gastric inflammation [12].

H. pylori is a relevant health problem among developing countries and few data concerning correlation between bacterial virulence factors and clinical outcomes is known in South America, particularly in Chile, which is a unique site for these studies, comparing outcomes and bacterial factors in host living in different regions because differences of *vacA* genotypes distribution in *H. pylori* along the country [13].

Because universal eradication therapy is not feasible and *H. pylori* vaccine it is not available at this point, it is of the utmost urgency to acquire a better understanding of the role of some virulence factors in the infection caused by this pathogen and the identification of mechanisms that regulate the immunological balance between host and *H. pylori* and their

interactions to improve diagnostic and therapeutic modalities in our country. The aim of this study was to evaluate how selected strain virulence factors and selected host immune response factors relate using a preliminary model based on data collected from infected patients living in different Chilean cities.

2. Materials and methods

2.1 Collection of clinical specimens

Twenty patients from Iquique (IQ), five from Santiago (SA), five from Temuco (TE) and fifteen from Punta Arenas (PA) were enrolled in this study with an age (mean ± SD) of 27.7 ± 18.9 years. Criteria for patient inclusion included symptoms suggestive of peptic disease (nocturnal or burning abdominal pain, chronic vomiting or hematemesis) or a history of recurrent abdominal pain plus a first-degree relative with an endoscopically proven diagnosis of peptic ulcer disease. Exclusion criteria were hemodinamically unstable patients and recent therapy with antibiotics (1 month). Recluted patients were in the pediatric as well as in the adult age range. A detailed clinical history was obtained that included the characteristics of the abdominal pain as well as other clinical manifestations, family history of peptic ulcer disease, and recent therapy with non-steroidal anti-inflammatory drugs, bismuth compounds or acid suppressing drugs. *H. pylori* clinical strains were isolated from gastric biopsies obtained from patients who underwent upper gastrointestinal endoscopy for medical indication after informed consent. For culture purposes biopsies were transported to Santiago in BHI glycerol medium, for ELISA purposes biopsies were transported as frozen samples in dry ice and immediately suspended in normal saline (750 μl). Samples were manually grinded with a polished conical glass that fits in Eppendorf tubes.

A patient was considered infected if the rapid urease test (HE-PY test, Bios Chile, Santiago) was positive and histological analyses of at least 1 out of 2 biopsies revealed the presence of Gram-negative rods (Warthin-Starry silver stain or H&E stain). We confirmed this assignment by isolation of colonies. Four (8.9%) of the patients were considered *H. pylori* negative (control group), 31 (68.9%) had histological chronic gastritis and 10 (22.2%) had duodenal ulcer.

2.2 Growth conditions and identification of *H. pylori* strains

H. pylori clinical strains were isolated from biopsies and colonies were grown in a microaerophillic atmosphere (5% CO_2) for 3–4 days on tryptic soy or Brucella agar plates containing 5% fresh horse blood, 5 mg vancomycin, 2.5 mg cefsulodin, 2.5 mg trimethoprime lactate, and 2.5 mg amphotericin B per 100 ml of medium. Single colonies were transferred to plates with the same medium but without horse blood. Morphologic characteristics were observed in both media. *H. pylori* were further identified based on biochemical tests for catalase, oxidase and urease [14].

2.3 Isolation of H. pylori chromosomal DNA

Cells coming from half of agar plate were collected and transferred to an Eppendorf tube using a sterile loop and processed as described by Owen and Bickley, with CTAB as detergent for cell lysis to obtain chromosomal DNA [15].

2.4 PCR reactions, primers, cloning and sequencing

PCR was used to amplify the *cagA* and *vacA* gene. The primers were designed according to the 26695 strain [16] and the Chilean strain CHCTX-1[17]. The primers used for *cagA* amplification are shown in table 1. The A17BN1 and Cag6 primers were used to amplify from the beginning of A17 central region of cagA fragment up the 3′ (C- terminus) of the *glr* gene (glutamate racemase gene). The A17BN1 and Cag7 primers were used to amplify from

the beginning of the A17 fragment to 3′ end of *cagA* gene. Finally, to ensure the presence of *cagA* gene, the region A17 was amplified by PCR using the primers A17BN1 and A17BH2 [18]. The PCR mixture (20 ul) consisted of 15–20 ng of chromosomal DNA, 0.5uM each primer, 0.2mM each dNTP, 50mM $MgCl_2$, and 0.5 Units of Taq DNA polymerase (Fermentas). The cycle conditions were 1 min at 94°C; 2 min at 50°C; and 3 min at 72°C for 30 cycles. A final elongation step of 5 min at 72°C was added. Amplifications were done in a PTC-100 MJ Research apparatus. As a PCR positive control, a 400 bp fragment of *tsaA* gene (alkyl hydroperoxide reductase) from *H. pylori* was amplified under the same conditions described for *cagA* amplification, using the AG261 and AG26M primers (Table 1) [19].

Primers and reactions for the *vacA* amplifications were done according to the conditions established by Atherton et al [20]. Cloning of s1a, s1b, s2, m1 and m2 PCR fragments from the isolates were done by ligation into pGEM-T easy vector (Promega) and electroporation in *E. coli* strain DH5α plated on Luria agar containing 50μg/ml of Ampicilin. The sequences were done in ABI Prims-3100 genetic analyzer (Apllied Biosyztems). The sequences of the fragments were published in the service of the National Library of Medicine and the National Institute of Health (pubmed central) in a previous work done by our group [13]. *Campylobacter yeyuni* ATCC 29428 was used as DNA source in negative control samples and the strains ATCC 26695, ATCC 43504, J99, CHCTX-1 [17], 8823 and 8822 were used as positive control of *vacA* PCR amplification.

2.5 Cytokine determinations assays

For cytokine determination, the remaining antral biopsy specimens were homogenized with a tissue tearor (OMNI Th international) separately in 750 μl of normal saline. Supernatants obtained by centrifugation in a mini Eppendorf centrifuge (12,000 g for 5 minutes at 4°C) were frozen at −20°C in sterile vials (Sardstest) until used for an enzyme-linked immunosorbent assay (ELISA). Protein was measured using a modified bicinchoninic acid method (Pierce, Rockford, IL) and the total protein concentration in biopsy homogenates was expressed as mg/ml. The range of detection was between 0.02 and 2 mg/ml. IL-12, IL-10, IFN-γ and IL-4, were measured by ELISA (BD Biosciences Pharmingen, San Diego, CA) on supernatants of homogenates, as recommended by the manufacturer using recombinant human cytokines as positive controls for the development of standard curves. These assays demonstrated no measurable cross-reactivity to other cytokines and the limits of detection were 4.0 pg/ml for IL-12, 2.0 pg/ml for IL-10, 1.0 pg/ml for IFN-γ, and 2.0 pg/ml for IL-4. The final cytokine concentrations in biopsy homogenates were expressed as pg/mg of protein.

2.6 Statistical analysis

Chi square, Mann-Whitney, Kruscall Wallis and Dunn post test were used for comparison between groups. A multiple regression analysis was performed using *H. pylori* genotypes of *cagA* and *vacA*, levels of cytokines, age, gender and city of origin of the patients as explicatory variables of clinical outcome. The variables or interactions that did not contribute significantly to the model were excluded to reduce the model to a minimum number of interaction terms. A p value <0.05 was considered statistically significant.

3. Results

3.1 Bacterial virulence factor

Eighty four percent of *H. pylori* infected patients included in this study were positive for *cagA* gene (Table 2). The size of the fragments amplified from strains used in this study were according to those expected for the strain 26695 (3500 bp for A17BN1-Cag7 primers

and 3000 bp for A17BN1-Cag6 primers and 1400 bp for A17 fragment, Figure 1). 91.2% were positive for *vacA* gene. The alleles s1bm1, s1am1 and s2m2 alleles were detected in 78%, 11% and 2.2% of the cases, respectively (Table 2). Fig. 2 shows PCR reactions for the different *vacA* alleles, according to the conditions described in method section. Virulence factors were present in infected patients regardless of severity of clinical outcome and there was no significant difference in *cagA* and *vacA* status in patients with chronic gastritis and duodenal ulcer (Table 2).

3.2 Levels of cytokines

Individual cytokines such as IL-10, IL-12, IFN-γ and IL-4 showed no statistical difference among patients with different clinical outcomes (Table 2). The ratio of cytokines was used for assessment of T helper profile. IFN-γ/IL-4 ratio showed a non significant increase in patients with chronic gastritis or duodenal ulcer in comparison to patients diagnosed as *H. pylori* negative (Fig 3A). IL-12/IL-10 ratio increased in parallel with the severity of clinical outcomes showing significant differences between non infected patients and patients with duodenal ulcer disease (p=0.04, Fig. 3B).

3.3 Correlation between host and bacterial factors

By multiple regression analysis we obtained a model that considered that clinical outcome to *H. pylori* infection, such as chronic gastritis or duodenal ulcer, was determined by the interaction of host immune response measured by Th cytokine balance and bacterial virulence factors, such as CagA but not VacA. This interaction was defined by the equation where Clinical outcome = 0.867491 (*cagA*) + 0.0131847 (IL-12/IL-10) + 0.0103503 (IFN-γ/IL-4). Variables included in this equation had a p value of less than 0.01 showing a statistically significant relationship between them. The model had a R-squared of 0.964 explaining 96.4% of the variability in clinical outcome.

To determine the accuracy of our equation in relation to the endoscopic diagnosis of duodenal ulcer we obtained theoretical titers by fitting cytokine profile and *cagA* status for each patient. Duodenal ulcer showed a significant increase of equation titers from chronic gastritis to duodenal ulcer (p=0.01, Fig 4).

4. Discussion

The present study investigates the relationship between selected bacterial virulence and host immune response on the clinical outcome of *H. pylori*-associated disease. Substantial morbidity is associated with *H. pylori*-induced diseases; approximately 90–95% of duodenal ulcers and 70–75% of gastric ulcers are attributable to this infection [21]. Therefore it has been considerable efforts focusing on delineating the complexes mechanisms by which this pathogen induces gastric inflammation and disease.

The relationship between the *cag* Pathogenicity Island and peptic ulcer are inconclusive. There are a number of studies showing that patients with duodenal ulcers have increased frequency of infection with cag^+ strains [22–24]. However, in different populations the frequency of cag^+ strains is highly variable, and it is not surprising that in populations with a high frequency of cag^+ strains, the association with ulceration has not been observed and the associated risk for peptic ulcer is the same for *H. pylori* infection regardless the *cagA* status [25–27]. Previous results of our group showed prevalence of 84% of CagA positive patients measured by antibody titers [30]. In the current study infected patients showed 84 % positivity for *cagA* measured by PCR reaction with an almost identical extent of the *cagA* gene in patients with higher levels of inflammation or mucosal damage, such as duodenal ulcer patients than in patients with gastritis, showing no capability for discrimination between clinical outcomes by itself.

VacA gene, which encodes a secreted bacterial cytotoxin, has also been involved in the pathogenesis of peptic ulcer disease. Although *vacA* gene is present in virtually all *H. pylori* strains they vary considerably in their cytotoxic activity due to variations in *vacA* gene structure [5]. Strains that possess an s1/m1 *vacA* genotype are considered as most virulent with an increased risk of peptic ulcer disease and gastric cancer and enhanced gastric epithelial cell injury compared to those that possess s2/m2 alleles [24]. Prior work from our group showed that the most prevalent VacA genotype in Chilean patients was s1bm1 with 76%, followed by s1a m1 with 21% and s2 m2 that occurred in only 3% [13]. In the current study 91.2% of *H. pylori*-infected patients were carrying VacA positive strains also with higher proportion of s1bm1 genotype. Because the presence of *cagA* and *vacA*, particularly virulent alleles like s1m1, is so extended in our population, and ulcer percentages is not particularly high, we were not able to use these virulence markers as sole predictors of duodenal ulcer disease. Similar results were observed in a Japanese population which had a 79% of positivity for type I strain (CagA and VacA positive) showing that in high prevalence countries the presence of virulence factors can not be used as predictors of different outcomes on their own [28].

The host immune response is an important determinant of *H. pylori*-associated disease. *H. pylori* colonization induces a systemic and mucosal response directed to multiple antigens [29]. Among the local immune mediators T helper cytokines may play a pivotal role in pathogenesis in patients with different *H. pylori*-associated outcomes. Individual cytokine levels are not related with clinical outcomes in this study. However, there is an increase of the Th1 response, as measured by IL-12/IL-10 ratio, as mucosal damage progresses from chronic gastritis to duodenal ulcer. This predominant Th1 response observed during infection is unlikely to be effective in eradication of the pathogen and may contribute to gastroduodenal pathogenesis [12]. However, the Th1-predominant response is not able to explain all the variability of our subjects in terms of *H. pylori*-associated morbidity.

Although the presence of *cagA* and *vacA* genes in Chilean population are equally extended and the deviation towards a Th1 type response in peptic ulcer patients is not consistent for all measured cytokines, the development of duodenal ulcer disease is associated mainly to the presence of CagA and to the Th1/Th2 cytokine ratios. Our model accounts for 96% of our sample variability and is able to discriminate between patients with mild inflammation (gastritis) from those who develop severe outcomes such as duodenal ulcer.

The present study demonstrates that the relationship between bacterial virulence and host immune factors is determinant in the development of clinical outcomes of *H. pylori*-associated diseases. Our results show that in countries with high prevalence of *H. pylori* infection with virulent strains, we can not consider virulence factors and host immune response to be unconnected processes, so for analytical approximations to predict or describe the appearance of diverse outcomes in *H. pylori* infected patients, a multivariate course of action must be considered.

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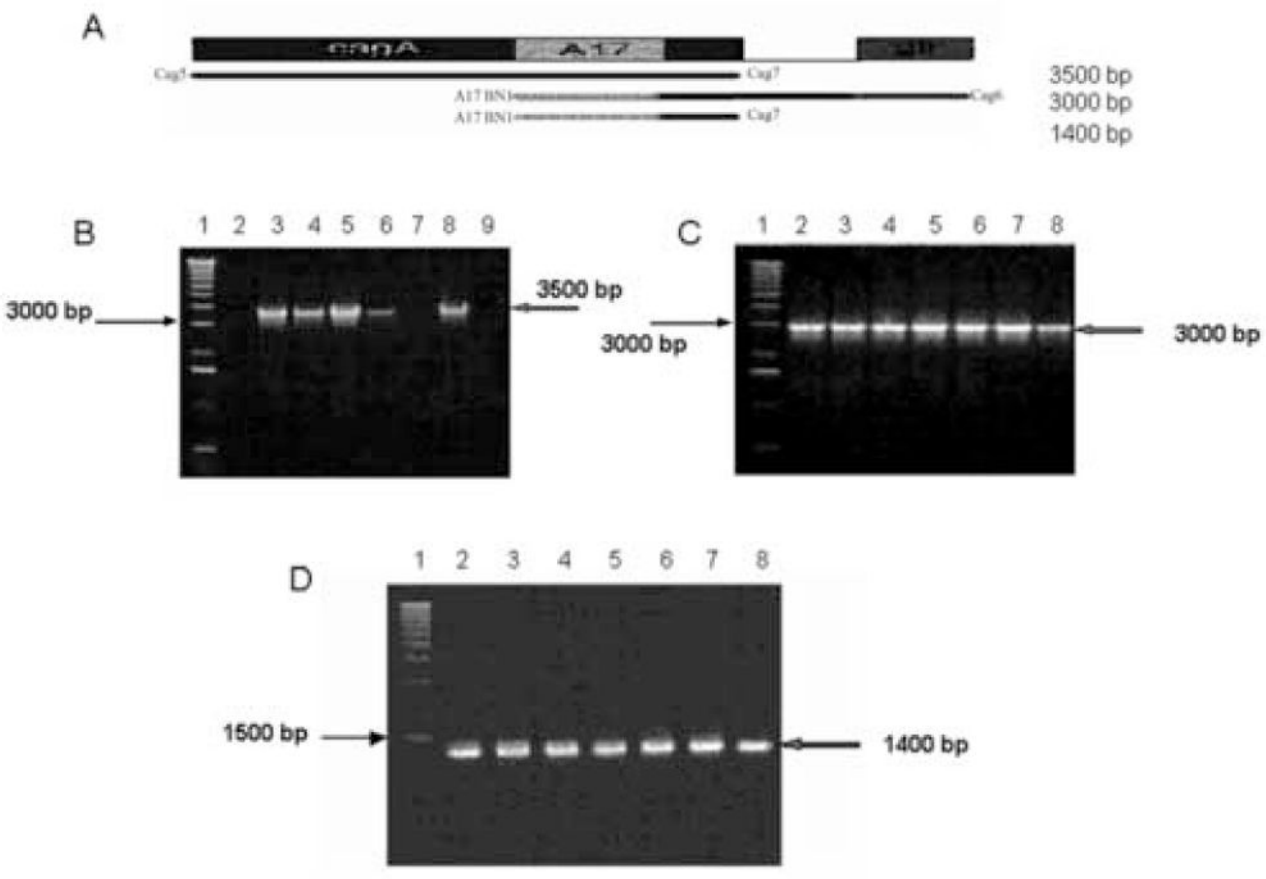

Figure 1.

*cag*A detection. A. Scheme showing the amplified region of *cagA* gene. The primers were designed according to the 26695 strain as described in the Materials and Methods section. B. Amplification using A17BN1-Cag7 primers. Lane 1: 1kb DNA ladder Gibco. Lane 2: strain cagA -. Lanes 3–6 positive samples for this reaction. Lane 7: negative control with template DNA isolated from *Campylobacter jejuni*. Lane 8: positive control with chromosomal DNA isolated from strain 26695. C. Amplification using A17BN1-Cag6 primers. Lane 1: 1kb DNA ladder Gibco. Lanes 2–7: positive samples for this reaction. Lane 8: positive control with chromosomal DNA isolated from strain 26695. D. Amplification using A17BN1 and A17BH2 primers. Lane 1: 1kb DNA ladder Gibco. Lanes 2–7: positive strains for this reaction. Lane 8 positive control with chromosomal DNA isolated from strain 26695. Arrows indicate the expected size for each fragment and the size of the nearest band in DNA ladder.

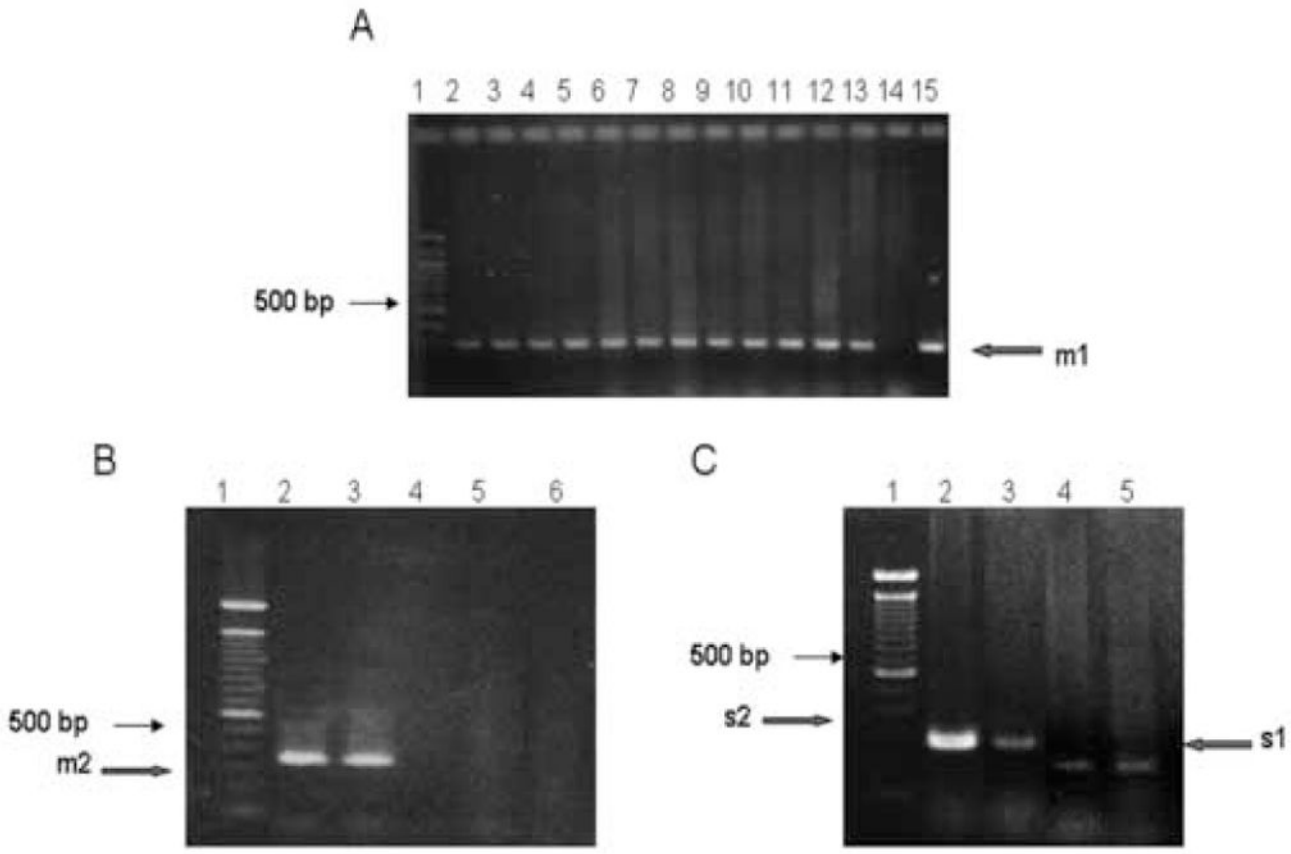

Figure 2.

*vac*A amplification. A. PCR amplification of m1 allele. Lane 1: 100 bp DNA ladder standard, Lanes 2 through 13 samples obtained from patients living in IQ. Lane 14: negative control with template DNA isolated from *Campylobacter jejuni*. Lanes 15: positive control with chromosomal DNA isolated from strain J99. The arrow indicates the 290 bp expected PCR product. All PCR assays were repeated at least 3 times. B. PCR amplifications for m2 VacA allele. PCR fragments were separated in a 3% agarose gel. Lane 1: 100 bp ladder DNA standard, Lane 2: positive sample from a patient living in SA. Lane 3: positive control with chromosomal DNA isolated from strain 8822. Lanes 4 and 5: negative samples. Lane 6: strain 26695 as a negative control. The arrow indicates electrophoretic migration in a 3% agarose gel of the 352 bp expected fragment. C. Size differences in s1 and s2 PCR fragments derived from vacA gene from Chilean *H. pylori* clinical isolates. Fragments were separated in a 3% agarose gel. Lane 1: 100 bp ladder DNA standard. Lanes 2 and 3: s2 positive strains isolated from SA. Lane 4: a s1 positive strain from TE. Lane 5: a S1 positive control corresponding to *H. pylori* strain 26695. Arrows indicate the expected size for each fragment.

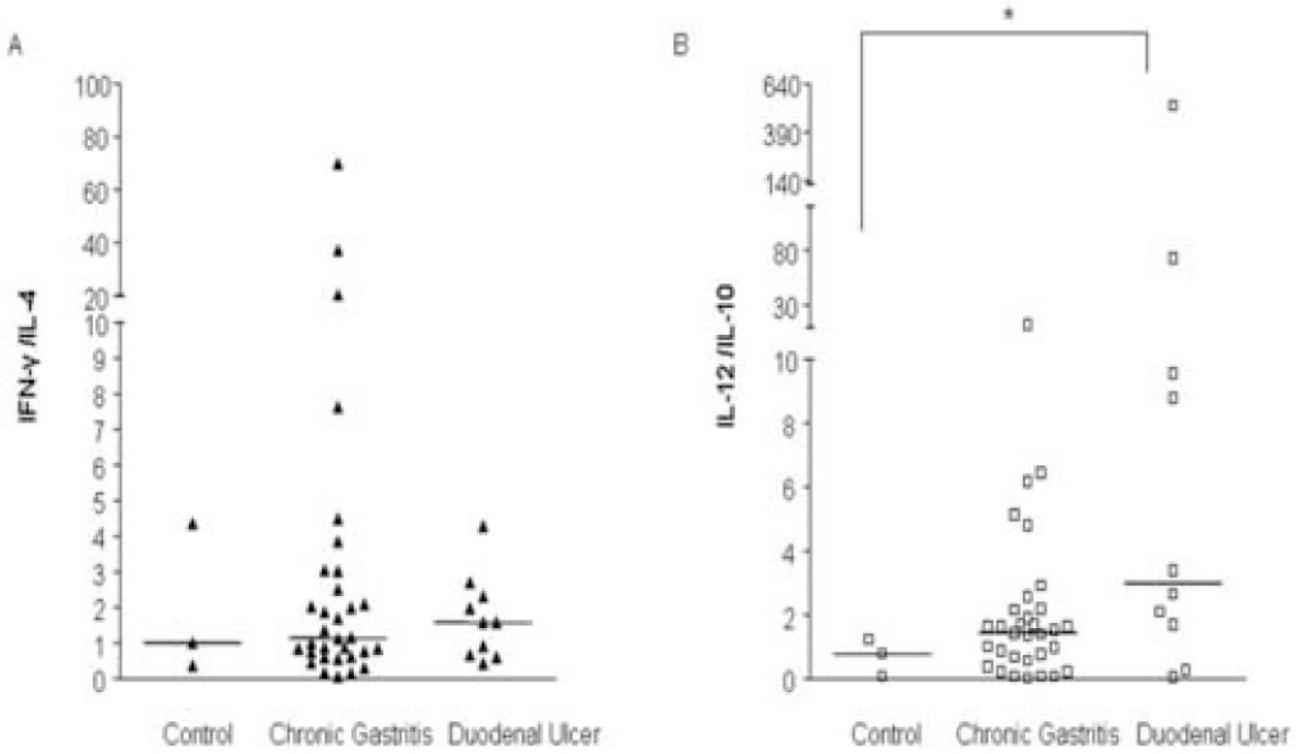

Figure 3.

A. IFN-γ/IL-4 ratio showed no statistical difference among patients with different clinical outcomes. B. IL-12/IL-10 ratio was higher in patients with duodenal ulcer compared to non-infected patients. Data are presented as individual points plus median. $p<0.05$, Kruscall-Wallis, Dunn post test.

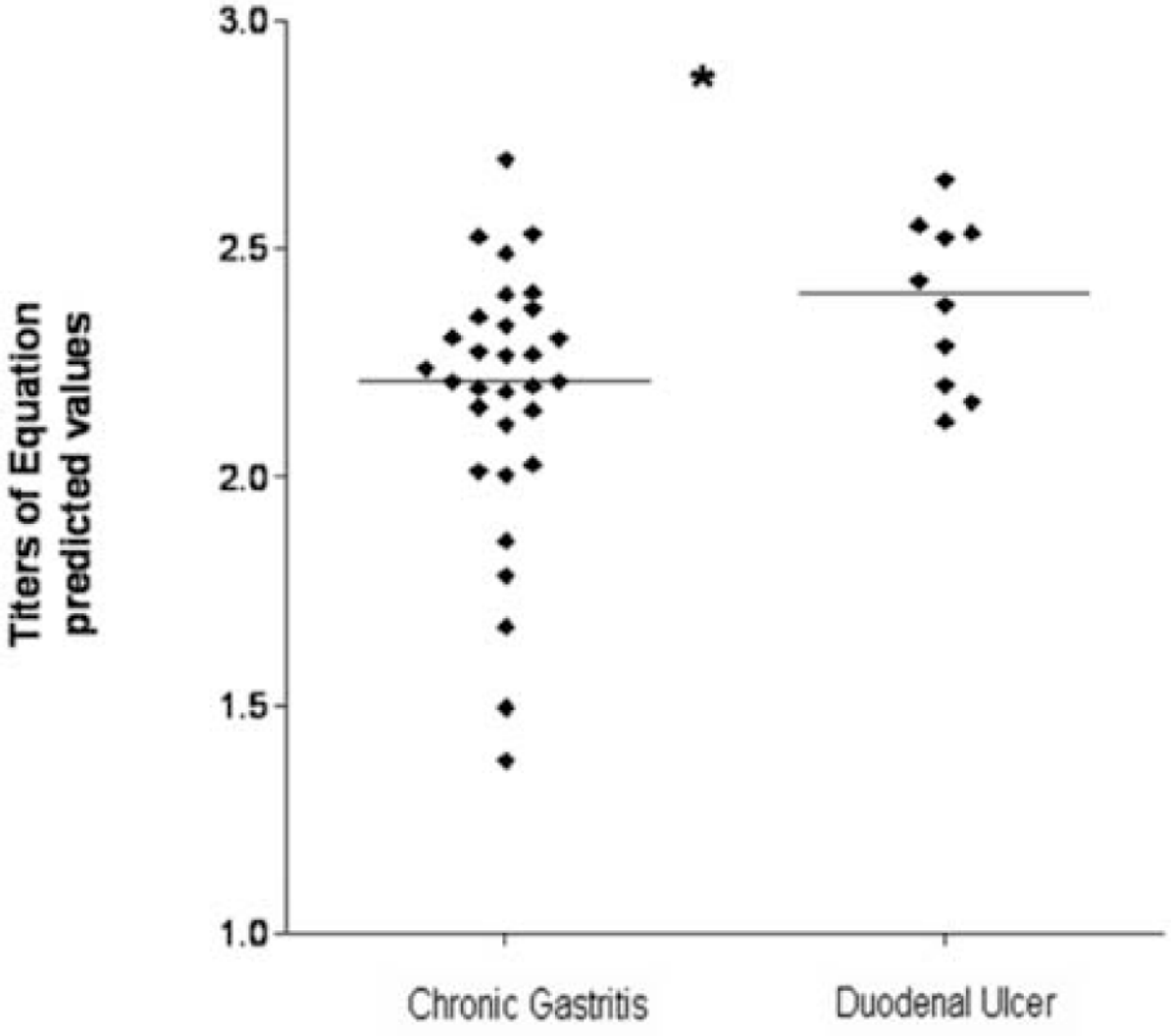

Figure 4.

Observed and predicted values of clinical outcomes explained by the equation Clinical outcome: 0.867491 (*cagA*) + 0.0131847 (IL-12/IL-10) + 0.0103503 (IFN-γ/IL-4) that relates virulence factors such as CagA and host immune response defined as Th1/Th2 cytokine ratios. Titers of equation predicted values, obtained by fitting to the equation measured parameters, increase with severity of endoscopy observed outcome. Data are presented as individual points plus median. $p<0.05$, Mann-Whitney test.

Table 1

Primers used in PCR reactions for *cagA, vacA* and *tsaΔ* amplifications

Name	Sequence 5′→3′
A17BN1	agatctcatatgaaaaatggcaaaaataaggatttcagcaag
A17 BH2	ggatccaagctctattattctgataccgcttgattgagattgtc
CagA5	taaggagaaacaatgactaacgaa
CagA6	atgaaaataggcgttttgatagcggt
CagA7	ttaagatttttggaaaccacctttgt
A6261 *	tgcggatccatatgttagttacaaaacttgcccccgat
A62M *	taaataagcttctctcaaagcgatcgcttcttcaaacagcacatc
VA3F **	ggtcaaaatgcggtcatgg
VA3R	ccattggtacctgtagaaac
VA4F **	ggagccccaggaaacattg
VA4R	cataactagcgccttgcac
VA1F **	atggaaatacaacaaacacac
VA1R	ctgcttgaatgcgccaaac
VA1F **	atggaaatacaacaaacacac
VA1R	ctgcttgaatgcgccaaac
SS1F **	gtcagcatcacaccgcaac
VA1R	ctgcttgaatgcgccaaac
SS3F **	agcgccataccgcaagag
VA1R	ctgcttgaatgcgccaaac
SS2F **	gctaacacgccaaatgatcc
VA1R	ctgcttgaatgcgccaaac

* Primers used for amplification of the *H. pylori tsa*A gene.

** Primers pairs used for amplification of different regions of *H. pylori vac*A gene

Table 2

Characterisitics of the study groups

N [%]	**Chronic Gastritis 31 (68.9)**	**Duodenal Ulcer 10 (22.2)**
Age, X ± SD	27.1 ± 21.3	34.4 ± 15.1
Male, N° [%]	9 (29)	6 (60)
***H. pylori* infected, N° [%]**	31 (100)	10 (100)
Presence of CagA, N° [%]	28 (90.3)	10 (100)
Vac Alleles, N° [%]		
-s1bm1	27 (87.1)	8 (80)
-s2m2	1 (3.2)	0 (0)
-s1am1	3 (9.7)	2 (20)
Cytokines, median (interquartile range)		
-IL-10	24.5 (32.5)	13.8 (24.9)
-IL-12	21.7 (53.4)	23.5 (65)
-IL-4	14.2 (24.7)	36.9 (41)
-IFN-γ	12.6 (27.6)	25.4 (40.5)

p=ns