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Prevalence of serum antibodies to *Helicobacter pylori* VacA and CagA and gastric diseases in Chile

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The objective of this study was to evaluate the prevalence of antibodies to *Helicobacter pylori* CagA and VacA proteins and correlate this prevalence with gastric diseases in colonised Chileans. The study was performed in 418 adults colonised with *H. pylori*: 316 with gastroduodenal pathology (152 duodenal ulcer, 14 gastric cancer and 150 gastritis patients) and 102 asymptomatic subjects. Serum IgG antibodies to *H. pylori* were determined by enzyme immunoassay (EIA). Antibodies to VacA and CagA proteins were detected by Western blotting. In a subgroup of the patients, the vacuolating activity was determined by HeLa cell assay and the CagA product was confirmed by PCR assay. IgG antibodies to both VacA and CagA proteins of *H. pylori* were found in 270 (85%) of 316 colonised gastric patients and in 72 (71%) of 102 asymptomatic subjects. Colonisation with virulent strains was significantly higher among duodenal ulcer and gastric cancer patients than in gastritis patients or asymptomatic subjects. Infections with $VacA^+$/$CagA^+$ *H. pylori* strains is common in Chile but, in contrast to some Asian countries, this phenotype was more prevalent in isolates from patients with more severe gastric pathologies.

Introduction

In a relatively short time-span, *Helicobacter pylori* has become recognised as a major gastric pathogen with a worldwide distribution. It is estimated that the prevalence of *H. pylori* infection ranges from 15% to 90% of the population in both developed and developing countries [1–4]. The pathogenic role of *H. pylori* in type B chronic gastritis, peptic ulcers and gastric cancer is well documented [5, 6]. Infection usually occurs without overt clinical symptoms, particularly in poor communities [4, 7]. However, environmental factors are not unique in determining the clinical impact of *H. pylori* infection in a given population, as the host's immune status and some virulence characteristics of the infecting strains [8, 9] appear to influence the severity of clinical symptoms.

There is sufficient evidence to implicate expression of the bacterial products VacA and CagA proteins as two major determinants of virulence of *H. pylori* [10, 11]. VacA is a potent cytotoxin (*c.* 87 kDa) that induces intracellular vacuolation in various human cell lines *in vitro* [12]. CagA is a high-mol.-wt (*c.* 130 kDa) virulence marker for the pathogenicity island, *cag*, and *cagA*-positive strains induce local epithelial cells to release cytokines, i.e., (IL) interleukin-8, IL-6 and tumour necrosis factor (TNF)-α [13, 14]. It is estimated that 40–60% of the *H. pylori* strains isolated from duodenal ulcers and 30% of the strains isolated from type B gastritis are cytotoxic [10, 15–17]). Several studies have reported that *cagA* is present in 70–90% of clinical isolates of *H. pylori* [15, 18–20].

Serological methods have been used to determine *H. pylori* colonisation in epidemiological studies [21, 22] and to monitor the success of triple therapy [23, 24]. Several reports from developed countries [10, 11, 15, 21] have suggested that antibodies to CagA protein are present at significantly higher frequencies in peptic ulcer patients than in those with chronic gastritis or in healthy colonised individuals. According to these reports, colonisation by $CagA^+$ strains represents an increased risk of developing severe gastric pathology. However, results of studies in China [20], Korea [25] and Japan [26] question this hypothesis, as in these countries 90% of *H. pylori* strains are VacA- and CagA-positive. These and related results have suggested that there is no correlation between VacA and

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CagA status and the patient's clinical pathology in some geographical areas. Thus, colonisation by $VacA^+$ $CagA^+$ *H. pylori* strains could not be widely used as a marker of risk for developing severe gastric pathologies.

As there is little information on this issue in South America, this study sought to determine the prevalence of antibodies to CagA and VacA in serum samples from Chilean subjects colonised with *H. pylori* and to correlate these findings with the presence of gastric pathology.

Materials and methods

Study design

Antibodies to VacA and CagA were determined by Western blotting with a panel of sera from Chilean individuals colonised with *H. pylori*. Specific IgG antibodies to VacA and CagA were detected by their ability to recognise 87- and 128-kDa protein bands in the Western blot, respectively.

Serum samples were collected between 1995 and 1998 from 418 individuals; 316 were patients who required upper gastrointestinal endoscopies and 102 were asymptomatic blood donors enrolled during their annual health check-up. The 316 gastric patients included 152 cases with duodenal ulcers, 150 cases with type B gastritis and 14 patients with confirmed gastric carcinoma. *H. pylori* infection in gastric patients was evaluated by analysis of gastric biopsies, urease test, histology and culture. A patient was considered to be colonised when all these parameters were positive. Colonisation of asymptomatic subjects was assessed by the determination of specific IgG antibodies to *H. pylori* (EIA and immunoblot). None of the patients was taking antisecretory agents when endoscopy was performed, or in the preceding 2 weeks. During endoscopic examination of the gastric patients, antral biopsy specimens were obtained for detection and isolation of *H. pylori*. A blood sample (5 ml) was taken from each patient before endoscopy for immunoassays and Western blotting. Serum samples were centrifuged and stored frozen (−40°C) in small volumes until EIA or Western blots were performed.

Informed written consent was obtained from each individual in accordance with the guidelines of the Institutional Ethics Committee. Alcoholic subjects, subjects with chronic diseases, pregnant patients, those with immune deficiencies and subjects taking antibiotics or non-steroidal anti-inflammatory drugs were excluded from the study.

Immunoassays

The presence of IgG antibodies to *H. pylori* was determined by an in-house EIA (99% sensitivity and 85% specificity) [27] with a surface antigen [28] obtained from a pool of *H. pylori* strains isolated in this laboratory (six from duodenal ulcer and four from gastritis patients). The antigen was prepared by acid glycine (0.2 M, pH 9.6) extraction for 15 min, followed by centrifugation at 11 000 *g*. The supernate was neutralised and dialysed. Briefly, microtitration plates were coated with *H. pylori* surface antigen (0.45 μg/well) and blocked with PBS-Tween with skimmed milk 10%; sera were added and the plates were incubated for 2 h at 37°C. After washing, goat anti-human IgG alkaline phosphatase conjugate (Sigma) was added and incubated for 1 h at 37°C. Wells were washed, and for colour development the substrate (p-nitrophenyl phosphate) was added and the EIA plates were incubated for 30 min at 37°C. The reactions were stopped with 3 N NaOH. Results were expressed as optical density (OD) at 405 nm. The cut-off value was calculated on the basis of the OD readings (mean + 2 SD) of the sera from 11 non-colonised adults, as confirmed by endoscopy and histological examination. Antibody titres were defined as the serum dilution that gave an $OD = 0.15 \pm 0.05$. To avoid inter-assay differences, control sera with known high and low titres were included in each experimental determination. Final results were the mean of duplicate experiments.

Immunoblots

H. pylori VacA and CagA status was determined serologically by immunoblotting the sera of patients against a soluble antigen of *H. pylori*. This assay was performed with an antigen prepared from the $VacA^+$ $CagA^+$ strain TC1, isolated locally from a patient with gastric cancer. The presence of the *cagA* gene was detected by PCR [29], and the $VacA^+$ phenotype was detected by the HeLa cell culture assay [10], by induction of the vacuolating effect. On the immunoblots, this strain exhibited the corresponding 87- and 128-kDa protein bands. The soluble cell antigens [30] were electrophoretically separated by SDS-PAGE, with a 4% stacking gel and 7% running gel. Proteins were transferred to nitrocellulose membranes according to Burnette *et al*. [31]. Briefly, strips were blocked with skimmed milk, treated with 1 in 150 serum dilutions and held overnight at room temperature. Membranes were then incubated with an anti-human IgG–alkaline phosphatase conjugate. Reaction was revealed with 5-bromo 4-cloro indoxyl phosphate, nitroblue tetrazolium and $MgCl_2$. The molecular masses of epitopes observed on the blots were calculated by interpolation in a curve constructed with reference markers (Gibco). Antibodies against 128- and 87-kDa antigens of *H. pylori* were detected.

The immunological determination of CagA/VacA phenotype was confirmed in a subset of 10 strains by PCR and HeLa cell vacuolation assays [10, 29].

Statistics

Tests of categorical data were compared by χ^2 or Fisher's exact tests. These analyses were performed with computerised software (EPI-INFO; version 5.01a); p values $\leqslant 0.05$ were considered significant.

Results

The specific EIA for *H. pylori* antibodies showed that the asymptomatic subjects and all of the colonised individuals had high levels of serum IgG against *H. pylori*.

The results obtained in the analysis of the serum samples by immunoblots from the patients and asymptomatic individuals included in this study are shown in Tables 1 and 2. Table 1 shows that a high percentage of patients, as well as healthy donors, had antibodies to *H. pylori* CagA and VacA proteins. Table 2 shows the assumed prevalence of VacA/CagA *H. pylori* phenotypes in patients with gastric symptoms, as well as in asymptomatic subjects, as estimated by the presence of specific antibodies to these proteins in Western blot analysis. The high prevalence of both anti-VacA and anti-CagA antibodies in patients with gastroduodenal pathology (270/316, 85%), as well as in asymptomatic adults (72/102, 71%), suggests that the $VacA^+/CagA^+$ phenotype is frequent in *H. pylori* strains infecting Chilean individuals (Table 2). Moreover, the prevalence of this virulent phenotype is significantly higher ($p < 0.0001$) in duodenal ulcer and gastric cancer patients than in chronic gastritis patients or in asymptomatic controls.

Discussion

Previous studies performed on the Chilean population have revealed that *H. pylori* infection is common in asymptomatic individuals, adults and children [1, 32, 33]. Results indicate that a high proportion (75%) of Chilean adults (⩾35 years old) have serum IgG antibodies to *H. pylori* [27]. These findings correlate well with the high incidence of peptic ulcer disease and gastric cancer rates reported in Chile [34, 35]. Both conditions have a great impact on public health and further clarification of the pathogenic mechanisms involved is warranted.

The role of VacA and CagA proteins in the virulence of *H. pylori* has been well established *in vitro* and in epidemiological studies [10–12, 15]. In this study, the prevalence of antibodies to VacA and CagA in a population of 418 infected Chilean individuals was determined. The results showed that patients with peptic ulcers or gastric cancer had a significantly higher prevalence of antibodies to VacA and CagA than asymptomatic individuals or those with gastritis, which agrees with reports from developed countries [10, 11, 15, 19, 36]. The results of the present study also show that these antibodies were detected in a high percentage of all groups studied, as has been reported in Asia. However, the data presented here differ from those previously reported in Asian countries, where antibodies to VacA and CagA proteins were detected with similar high rates in symptomatic and asymptomatic persons [20–26]. Thus, immunoblot studies conducted by Ogura *et al.* [26] in Japan showed that antibody rates to a recombinant VacA protein were similarly high among patients with duodenal ulcer (95%), gastric ulcer (85%), chronic gastritis (95%) and

Table 1. Presence of *H. pylori* antibodies to CagA and VacA in patients and asymptomatic Chilean individuals

	Number (%) of individuals with antibodies			
Antibody against	Gastric cancer (n = 14)	Duodenal ulcer (n = 152)	Gastritis (n = 150)	Asymptomatic subjects (n = 102)
$CagA^{+*}$	14 (100)	147 (96)	122 (82)	76 (75)
$VacA^{+\dagger}$	13 (93)	143 (94)	117 (78)	72 (71)

*Gastric cancer-duodenal ulcer *vs* gastritis, $p < 0.00001$, χ^2 test.
†Gastric cancer-duodenal ulcer *vs* asymptomatic, $p < 0.00001$, χ^2 test.

Table 2. *H. pylori* virulence phenotypes recognised by serum antibodies from patients with different gastric conditions

	Number (%) of individuals with *H. pylori* phenotype				
Phenotypes	Gastric cancer	Duodenal ulcer	Gastritis	Asymptomatic	Total
$VacA^+CagA^+$	13 (93)	142 (93)	115 (77)	72 (71)	342 (82)
$VacA^+CagA^-$	0 (0)	1 (1)	2 (1)	0 (0)	3 (1)
$VacA^-CagA^+$	1 (7)	5 (3)	7 (5)	4 (4)	17 (4)
$VacA^-CagA^-$	0 (0)	4 (3)	26 (17)	26 (25)	56 (13)

$p < 0.00001$, χ^2 test, duodenal ulcer and gastric cancer *vs* gastritis and healthy controls.

endoscopically normal subjects (100%). Similar reports from China [20, 37] demonstrated that CagA antibody rates were equally high in gastric patients and in asymptomatic controls. According to the results of the present study, the prevalence of antibodies to VacA and CagA in Chileans is also high. However, in contrast to observations from Asia, significant differences were detected among symptomatic and asymptomatic populations. This is somewhat unexpected, because Chilean statistics on peptic ulcer and gastric cancer diseases more closely resemble those reported from Asian countries than those prevailing in the Western world.

In summary, these results have shown that sera from patients with duodenal ulcers and gastric cancer do recognise VacA and CagA epitopes with significantly higher frequency than those from counterparts with chronic gastritis or healthy controls. However, the high recognition frequency shown by sera from a majority of Chilean adults suggests that determining antibodies to VacA and CagA in colonised individuals is not a reliable indicator for selecting those at higher risk of developing severe gastric pathologies in this region. The characteristics of the host immune response or environmental factors may play an important role in the pathogenesis of peptic ulcer or gastric neoplasia. Also, this approach could be used to select and follow up asymptomatic individuals with lower risks of gastric pathology and help to identify other factors involved in the pathogenesis of gastrointestinal diseases.

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