Helicobacter pylori and Stomach Cancer: A Case-Control Study in Venezuela

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Abstract

The role of *Helicobacter pylori* infection in gastric cancer was evaluated in a high-risk population in Venezuela using serological assays in a study of 302 cases and 483 neighborhood controls. To investigate the claim that assays for *H. pylori* should use antigens derived from local strains, four different assays derived from Venezuelan and European strains were used. Prevalence of IgG *H. pylori* antibodies in controls was very high, with estimates between 72 and 92%. Prevalence was similar in cases and controls. However, cases had lower antibody titers. This effect was observed only in subjects with low pepsinogen (PG) levels PGI/PGII <3.0), which suggested that extensive atrophy in cases causes a loss of *H. pylori* infection, with a consequent reduction in antibody titer. In addition, advanced cases (stage II or higher) had lower antibody titers than less advanced cases, which indicated that the lower antibody titers in cases may be attributable partially to a diminished immune response. All of the four assays for anti-*H. pylori* antibodies gave similar results. No evidence was found for the superiority of the assay based on Venezuelan strains. These results are consistent with other case-control studies in high-risk populations and highlight the difficulties of investigating *H. pylori* infection in retrospective studies.

Introduction

In Venezuela, stomach cancer is the first cause of death from cancer in males and the third cause of death in females after cervix and breast. The cumulative mortality to age 74 in 1990 was 1.7% in males and 0.85% in females (1). The highest rates are found in the Andean region (states of Tachira, Merida, Trujillo, and Lara). In Tachira state, cumulative mortality to age 74 in 1993 was 4.1% in males and 2.2% in females. We have conducted the first case-control study of gastric cancer in this high-risk population. This report concentrates on the link between gastric cancer and the gastric bacterium *Helicobacter pylori*. Other risk factors will be reported in a forthcoming paper.

The wide geographical and temporal variations in gastric cancer incidence indicate the fundamental role of environmental factors. It has been proposed that infection with *H. pylori* is one of these factors. *H. pylori* is recognized as a cause of chronic gastritis, which may be regarded as the first step in a sequence of changes to the gastric mucosa possibly resulting in cancer (2). Acquisition of *H. pylori* occurs in childhood, with new adult infections being comparatively rare (3). This is in agreement with the results of migrant studies, which suggest that early life exposures are important (4). Moreover, the prevalence of *H. pylori* infection in successive birth cohorts is decreasing in developed countries (5–7), and this matches the long-term decline in gastric cancer rates (8).

In 1994, an international working group convened by WHO considered the available evidence as sufficient to classify *H. pylori* as carcinogenic to humans (9). In the same year, a NIH consensus panel concluded that the relationship between *H. pylori* and gastric cancer required further investigation (10). A recent comprehensive review is provided by Danesh (11). The strongest epidemiological evidence comes from the consistent findings of nested case-control studies, in which the blood samples used for *H. pylori* measurements were taken years before diagnosis. A combined analysis of these studies yields a risk ratio of 2.5 (95% CI,[2] 1.9–3.4; Ref. 11). However, among these studies there was an inverse relationship between the OR and the degree of adjustment for potential confounders, such as smoking and social class. Thus it cannot be excluded that the observed relationship is attributable to residual confounding. Evidence from retrospective case-control studies is weaker. In particular, there is a disparity between case-control studies in high-risk countries and low-risk countries. Few studies in developing countries show a positive association (12).

Although *H. pylori* infection is one of the most common infections in humans, the number of cases of gastric cancer is comparatively very small. This has prompted the search for cofactors for progression from *H. pylori* infection to gastric cancer. Among these, a classification of *H. pylori* strains has been proposed based on the presence (type I) or absence (type II) of a 40-kb pathogenicity island which makes type I strains more virulent (13). Because only type I strains have the gene for CagA, antibodies to CagA can be used as a marker of infection

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[2] The abbreviations used are: CI, confidence interval; OR, odds ratio; CagA, cytotoxin-associated antigen A; PG, pepsinogen; PGI, type I PG(s); PGII, type II PG(s); AU, arbitrary unit(s).

Table 1 Comparative results of four *H. pylori* assays using different antigens

Antigen	Prevalence		OR[a] (95% CI)
	Controls	Cases	
Dutch strains	83/119 (70%)	80/117 (68%)	0.77 (0.41–1.44)
Venezuelan strains	101/115 (88%)	91/104 (88%)	0.95 (0.42–2.2)
French strains	111/115 (97%)	87/104 (84%)	0.18 (0.05–0.51)
Commercial kit	160/173 (92%)	162/172 (94%)	1.25 (0.55–2.92)

[a] OR have been adjusted for age and sex.

Table 2 Quartiles of the ELISA index distribution among controls

Antigen	Lower quartile	Median	Upper quartile	Cutoff
Dutch strains	0.62	0.90	1.05	0.65
Venezuelan strains	0.49	0.83	1.05	0.21
French strains	0.39	0.77	1.09	0.21
Commercial kit	95.5	275.5	>300[a]	20

[a] Upper quartile is not known because values >300 are censored.

with type I strains. We have investigated the importance of *H. pylori* type by testing for antibodies to CagA.

Another possible consequence of *H. pylori* strain diversity is misclassification of infection status. A study in Thailand suggests that the sensitivity of an ELISA can be improved by using antigens derived from local strains of *H. pylori* (14). However, this finding was not reproduced in a study in China, which showed equally high sensitivity for ELISAs based on a pool of Chinese strains and a pool of United States strains (15). To investigate the effect of the antigens used in the assay, we have compared four different ELISAs using European and Venezuelan *H. pylori* strains.

Materials and Methods

Study Subjects. Briefly, a total of 302 cases of histologically confirmed stomach cancer were identified in the two main hospitals of San Cristobal, capital of Tachira state, from January 1991 to August 1997. Neighborhood controls were drawn for each case, matched on age (within 5 years) and sex. For the first 120 cases, one control per case was selected, and for the remaining cases, two controls per case were selected. Cases were classified according to the Laurén classification (16).

Specimen Collection. Blood (10 ml)was collected from cases and controls to measure *H. pylori* antibody levels and PG levels.

Laboratory Methods. *H. pylori* IgG antibody levels in sera were measured using four ELISA assays. The assays were performed during recruitment of cases and controls; thus, results are available on a subset of subjects. None of the assays were validated in our study population.

The first assay, conducted on the first 120 cases and controls, used a sonicate of bacteria isolated from patients from Amsterdam (17). The results were expressed in terms of an absorbance index (AI):

$$\text{AI} = \frac{\text{Patient's absorbance} - \text{mean absorbance of blank reading}}{\text{Reference absorbance} - \text{mean absorbance of blank reading}}$$

An absorbance index of 0.65 was taken as a cutoff point for positivity. This cutoff gave 95% sensitivity and 96% specificity in a validation study using Dutch patients referred for upper gastrointestinal surgery (17).

To investigate differences in antigenic properties between different strains, two additional ELISA tests were developed using Venezuelan strains and French strains and applied to the first 120 cases and controls. The assays were calibrated by using a reference serum included on each plate. The serum specimen and the reference specimen were assayed in triplicate. The results were expressed as an ELISA index obtained by calculating the ratio of the mean absorbance of the serum specimen to the mean absorbance of the reference specimen. A cutoff value of 0.22 was used to determine positivity. This cutoff was determined from the receiver operating characteristic curve in a sample of French patients, using histological diagnosis as a standard, and yielded a sensitivity of 88% and a specificity of 63% in the validation study.

The final *H. pylori* test was conducted on the first 173 cases and one of their controls using a commercial ELISA kit named "*Helicobacter pylori* IgG" (DIESSE Diagnostic Senese, Siena, Italy; Ref. 18). The results were expressed in AU. A cutoff of 20 AU was used to determine positivity based on a sample of Italian patients who were *H. pylori*-negative by histology and culture. Values >300 AU, which indicate very high antibody levels, were censored.

Anti-CagA antibodies were tested in the sera of all cases and controls, using a modification of the method described by Xiang *et al*. (19). Each serum sample was tested at 2-fold dilutions starting from 1:50. Results were given as "relative units" which were calculated by comparing the titers in the test sample with that obtained in a standard positive control. The cutoff level was determined at 20 relative units by testing repeatedly 20 samples from *H. pylori*-negative subjects.

Plasma PG levels were determined on all subjects by using PG-I and -II Riabead Kits (Dinabbot, Tokyo, Japan), a modified RIA method that has been previously described (20).

Statistical Methods. After classifying subjects as *H. pylori*-positive or -negative using the given cutoff points, ORs for *H. pylori* infection were estimated using logistic regression, controlling for age and sex. To avoid any arbitrariness in the choice of a cutoff point, the subjects were also divided into four groups of increasing antibody titers, using quartiles of the control distribution as cut points. A trend model was fitted using scores 1, 2, 3, and 4 for the quartile groups, and the results were expressed in terms of the OR between top and bottom quartile groups.

Results

Median age among cases was 63 with an interquartile range of 53–72 and an absolute range of 35–91. Seventy-one percent of the cases were male, and 29% were female.

Prevalence of IgG Antibodies to *H. pylori*. Prevalence estimates are given in Table 1. All of the assays gave high estimates for the prevalence of *H. pylori* infection, except for the first assay using Dutch strains. This difference is probably attributable to the use of a much higher cutoff point, because the range of values of the ELISA index is similar to the other noncommercial tests (Table 2).

For three of the assays, the prevalence was similar in cases and controls, but for the test using French strains, prevalence was substantially lower in cases than controls (OR, 0.18; 95% CI, 0.05–0.50).

Analysis by Antibody Titers. Table 2 shows the quartiles of the control distribution using the index produced by each ELISA. Histograms of the indices (not shown) indicated that none of the assays clearly discriminated the population into two groups that might be considered "positive" and "negative."

Table 3 ORs (95% CIs) for stomach cancer by quartiles of antibody titer, controlling for age and sex

H. pylori antigen	OR by quartile group (95% CI)				*P* for trend
	Q1	Q2	Q3	Q4	
Dutch strains	1.0	1.31 (0.61–2.80)	0.51 (0.22–1.19)	0.31 (0.12–0.78)	0.003
Venezuelan strains	1.0	1.10 (0.53–2.27)	0.64 (0.30–1.38)	0.64 (0.29–1.39)	0.13
French strains	1.0	1.05 (0.51–2.15)	0.47 (0.22–1.01)	0.24 (0.10–0.59)	0.0004
Commercial kit	1.0	1.61 (0.90–2.90)	0.79[a] (0.46–1.38)		0.14

[a] The upper two quartile groups were merged for the commercial kit because of the large number of subjects with ELISA indices >300, which were censored.

Table 4 Spearman's rank correlations between the absorbance values of four *H. pylori* assays

	Correlations			
	Dutch	Venezuelan	French	Commercial
Dutch strains	1.00	0.78	0.70	0.78
Venezuelan strains		1.00	0.71	0.68
French strains			1.00	0.58
Commercial kit				1.00

Table 5 *H. pylori* prevalence among cases by histological type and location of gastric cancer

H. pylori antigen	*H. pylori* prevalence		*P*
	Intestinal type	Diffuse type	
Dutch strains	41/61 (67%)	27/40 (68%)	1.00
French strains	46/57 (81%)	30/34 (88%)	0.52
Venezuelan strains	51/57 (89%)	29/34 (85%)	0.80
Commercial kit	82/88 (93%)	47/50 (94%)	0.86
	Cardia	Noncardia	
Dutch strains	9/13 (69%)	69/100 (69%)	1.00
French strains	9/11 (82%)	80/90 (89%)	0.61
Venezuelan strains	9/11 (82%)	76/90 (84%)	0.69
Commercial kit	22/22 (100%)	129/137 (94%)	0.60

Table 3 shows the ORs for gastric cancer by antibody titer, using the quartiles of the control distribution to divide the subjects into four groups. Both the Dutch strains and the French strains showed a strong negative dose-response effect that was highly statistically significant. A weaker negative trend was observed for the Venezuelan strains and the commercial kit.

Agreement between Different Assays. The four assays were compared using Spearman's rank correlation coefficient applied to the original absorbancy index. Results are presented in Table 4. Agreement between assays is generally good; correlations are in the range 0.7–0.8, with the exception of the correlation between the commercial kit and the test using French strains (0.58).

***H. pylori* Antibodies by Histological Type and by Site.** When cases were classified by histological type, the number of cases in each category was as follows: intestinal, 164 (55%); diffuse, 85 (28%); and other/unknown, 53 (17%). The proportion of *H. pylori*-positive cases was similar in both intestinal and diffuse cancers, as shown in Table 5. Cases were also classified by cancer site. The proportions were: cardia 41 (14%); noncardia, 241 (80%); and unknown, 20 (6%). Table 5 shows that a high prevalence of *H. pylori* was found in both cardia and noncardia cancers.

Antibodies to CagA. The estimated prevalence of antibodies to CagA was 79% in cases and 78% in controls. There was no evidence of a relationship between CagA positivity and gastric cancer (OR, 0.97; 95% CI, 0.68–1.39). Further analysis by quartile groups showed weak evidence of a negative trend ($P = 0.08$) with an OR of 0.74 (95% CI, 0.48–1.14) between the highest and lowest quartile groups.

Subgroup Analysis by PG Levels. One possible reason for the lower antibody titers in cases is the loss of *H. pylori* from the stomach because of extensive atrophy. To investigate this possibility, the subjects were divided into two groups according to their PG levels, because a low ratio of PGI:PGII is a marker of gastric atrophy (21). A cutoff value of PGI:PGII <3.0 was taken for the "low" PG group, with subjects above this threshold being considered to have "normal" PG levels. With this cutoff, 40% of the controls and 62% of the cases were in the low PG group.

When antibody levels were analyzed by subgroup, no significant trends were observed in the normal group, but strong and significant trends were observed for all of the assays in the low-PG group. The results are summarized in Table 6. A significance test for interaction between antibody titers and PG levels showed low *P* values ($P = 0.002–0.08$) for all of the assays except the one using Venezuelan strains ($P = 0.46$).

Cancer Stage. Another possible explanation for the lower antibody levels in cases is a reduced immune response in subjects with advanced gastric cancer. To investigate this possibility, the cases were analyzed by cancer stage. Tumor-node-metastasis stage data were available for only 237 cases. Among this group the stage distribution was 5% IA, 11% IB, 25% II, 35% IIIA, 1% IIIB, and 22% IV. A cancer stage of IA or IB was considered "early."

The cases were divided into quartile groups using the antibody quartiles in cases. All of the assays showed a significant association between antibody titer and stage, as shown in Table 7.

Discussion

The prevalence of *H. pylori* infection is high in both cases and controls, and the prevalence of type I (CagA-positive) *H. pylori* is also very high. The high seroprevalence of *H. pylori* antibodies is consistent with the results of a large endoscopic survey previously conducted in Tachira state, Venezuela, which showed an overall prevalence of *H. pylori* of 94% by histological diagnosis (22).

Although the high prevalence of *H. pylori* may possibly contribute to the high gastric cancer risk in this population, we have been unable to demonstrate a positive association between *H. pylori* and gastric cancer by comparing cases and controls within the population. In fact we have found a negative association. It is noteworthy that this negative association became evident only after careful examination of the data. In the original analysis, the subjects were divided into "positive" and "negative" groups and only the assay using French strains

Table 6 Subgroup analysis of relationship between antibody titre and gastric cancer by pepsinogen levels

Antigen	Normal (PGI:PGII >3.0)			Low (PGI:PGII <3.0)			Interaction *P*
	OR[a]	95% CI	*P*	OR[a]	95% CI	*P*	
Dutch strains	0.69	(0.20–2.34)	0.55	0.13	(0.03–0.48)	0.002	0.05
French strains	1.06	(0.30–3.80)	0.93	0.28	(0.09–0.90)	0.03	0.08
Venezuelan strains	0.93	(0.25–3.38)	0.91	0.31	(0.10–0.97)	0.04	0.46
Commercial kit	1.09	(0.51–2.33)	0.83	0.31	(0.14–0.68)	0.003	0.02
CagA	1.09	(0.48–2.48)	0.84	0.19	(0.08–0.47)	0.003	0.002

[a] OR between top and bottom quartile groups (baseline = bottom) according to a trend model, controlling for age and sex.

Table 7 Cases only: *H. pylori* antibody titres by cancer stage

Antigen and cancer stage	Quartile group				OR[a] (95% CI)	*P*
	Q1	Q2	Q3	Q4		
Dutch strains						
Early	4	6	13	10		0.007
					0.19 (0.06–0.65)	
Late	24	17	10	13		
French strains						
Early	4	5	13	10		0.009
					0.17 (0.04–0.63)	
Late	18	14	12	10		
Venezuelan strains						
Early	5	8	9	10		0.05
					0.28 (0.08–0.97)	
Late	20	11	14	9		
Commercial kit						
Early	4	6	5	14		0.01
					0.21 (0.06–0.72)	
Late	30	28	23	25		
CagA						
Early	5	11	8	15		0.04
					0.36 (0.14–0.95)	
Late	54	47	51	45		

[a] OR between top and bottom quartile groups (baseline = Q1) according to a trend model, controlling for age and sex.

showed an association with gastric cancer. When the results were analyzed by antibody titer, in subgroups defined by PG levels, the negative association was found for all of the assays in the subgroup with low PG levels (PGI:PGII <3.0). We believe that this negative association is not causal but is a consequence of the disease. Two factors seem to play a role in the reduced antibody titers in cases: loss of *H. pylori* from the stomach because of atrophy and reduced immune response.

Evidence that *H. pylori* is lost from the stomach in the precancerous stages of the disease comes from endoscopic studies. Karnes *et al.* (23) observed significantly lower prevalence of *H. pylori* (33%) by histological diagnosis than by serology (86%) in subjects with atrophic body gastritis. In an endoscopic survey in Italy, Farinati *et al.* (24) found that prevalence of infection increased with age but became significantly lower with the progression of gastric damage. The density of colonization followed the same trend. The same phenomenon has been observed in a large endoscopic study in Venezuela, in which histological diagnosis of *H. pylori* measured on a four-point scale (negative, difficult to find, easy to find, and abundant) was negatively correlated with degree of atrophy, intestinal metaplasia, and dysplasia (22).

Loss of *H. pylori* may also lead to a reduction in IgG antibody titer. Longitudinal studies in which *H. pylori* infection is eradicated by antibiotics show a substantial decrease in antibody titers 1 year after treatment (25, 26). Therefore, a reduction in bacterial load in the stomach during the decades before gastric cancer develops may lead to a reduction in plasma antibody levels.

The subgroup analysis by PG levels in this study gives direct evidence that gastric atrophy leads to a reduction in anti-*H. pylori* antibody titers. We found no relationship in the normal PG stratum but quite a strong relationship in the stratum with low PG levels. One possible explanation for this residual association is that low PGI:PGII is an indirect measure of atrophy and may have a higher positive predictive value in cases than controls because of a higher prevalence of extensive atrophy.

Indirect evidence for a reduced antibody response in cases comes from the correlation between stage and antibody titer among cases. Cases in the highest quartile group of antibody titer were more likely to be "early" (stage IA or IB) cases than those in the lowest quartile group. Another possible explanation is that advanced cases have fewer receptors for *H. pylori*, the *H. pylori* cannot attach themselves to the gastric cells, and the antibody levels decrease because there are fewer antigens.

We have not found any strong evidence of the superiority of the assay based on locally derived strains. The high correlations between the four different assays suggest that the antigenic properties of the different strains are not very important for serological diagnosis. The main difference in the prevalence estimates seems to be attributable to the choice of a much higher cut point for the assay using Dutch strains. We have used the cutoff points determined by external validation studies. Ideally, the assays should be validated in the study population, but, in practice, it is not possible to find a suitable control group of *H. pylori*-negative subjects with normal gastric mucosa in this population because of the very high prevalence of *H. pylori* infection.

Another important validity issue for *H. pylori* assays is the possibility of cross-reactivity with other bacteria such as *Campylobacter*. The assay using Dutch strains was tested for cross-reactivity in 10 positive sera for *Campylobacter jejuni* and no cross-reaction was observed. The commercial kit had been previously tested in 13 children with acute *Campylobacter jejuni/coli* diarrhea and only 1 of 13 samples tested positive (18). The assays using French and Venezuelan strains were not tested for cross-reactivity, but the results for the other assays suggest that cross-reactivity is not a major problem.

Our results suggest that case-control studies in populations with a high prevalence of *H. pylori* infection will yield little information about the relationship with gastric cancer. High prevalence in the general population means low power to detect a higher prevalence in cases. We have tried to avoid this problem by considering antibody titers, a strategy that has been used successfully to associate EBV with Hodgkin's disease (27) and Burtkitt's lymphoma (28) in populations with a high prevalence of EBV. However, this strategy cannot be successfully

used for *H. pylori* in retrospective studies of gastric cancer because it appears that the disease itself causes lowering of antibody titers.

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