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Helicobacter Pylori IgG Sero-Prevalence Study in Two Adjacent Regions of Contrasting Gastric Cancer Rates and Bracken Fern Frequency in Western Venezuela

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Abstract

As Human Gastric Cancer Death Rates (HGCDR) is unevenly distributed in western Venezuela, we explored possible risk factors, among which *Helicobacter pylori* (Hp) infection alongside bracken fern invasion could be differential contributors. A prospective transversal study for Hp seroprevalence was conducted in a large number of healthy individuals (15-65 years) in two geographically distinct zones (low megathermic and mountain mesothermic, 0-3000 meters) employing IgG Hp ELISA response at district level. Results were matched with HGCDR records. Hp prevalence was high and statistically similar ($p > 0.05$) in all populations of both sexes [lowlands: 85.0% ($CI_{95\%}$ 72.7-97.3); highlands: 87.2% ($CI_{95\%}$ 83.2-92.3); $p = 0.1138$], males more than females in both regions [$RR_{M/F}$: lowlands 1.17 ($CI_{95\%}$ 0.99-1.36); highlands 1.16 ($CI_{95\%}$ 1.06-1.25)], with the middle aged group being the most affected [lowlands: 94.6% ($CI_{95\%}$ 68.4-119.7); highlands: 86.0% ($CI_{95\%}$ 60.3-111.8)]. A clear HGCDR-elevation gradient was found in both sexes but no association with Hp prevalence. Differential bracken fern invasion in cattle farmland is proposed as a relevant HGCDR risk factor.

Keywords

Helicobacter pylori, IgG seroprevalence, ELISA, Gastric cancer, Bracken fern, Venezuela

Introduction

Essential for the comprehension of Human Gastric Cancer (HGC) epidemiology is the *Helicobacter pylori* (Hp) infection status of the HGC-affected populations ever since this bacterium acquired the status of major risk factor in serious stomach diseases [1]. These conditions include Non-Cardia Neoplastic Lesions (NCGC) which in turn is contingent on host genetic polymorphism, as well as epigenetic and environmental factors [2-4]. Supporting evidence stems from HGC Relative Risk (RR) estimates due to all *H. pylori* genotypes which fall in the range 2.8-6.0 among *H. pylori* positive (Hp(+)) patients as compared with Hp(-) people [5-7]. At the molecular level, Hp jeopardizes the integrity of the host genome [8], down-regulates the mismatch repair protein response, promotes Reactive Oxidative Species (ROS) DNA damage [9] and causes DNA strand breaks and repair responses in affected epithelial cells [10]. More detailed molecular oncogenic factors, gastric epithelial cells modifications and the role of Hp outer membrane proteins are being discovered in a stream of recent publications [11,12].

H. pylori are highly prevalent in developing countries (> 85% of the adult population). HGC is also a major cause of death in these countries as two-thirds of the 900,000 new cases registered yearly occur there [13]. Seroprevalence studies in Latin America reveal high *H. pylori* rates from childhood to adult age [14-19], reach-

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ing nearly 100% prevalence in HGC patients or suffering from other serious epigastric pathologies [20,21].

As part of a long term study, we became interested in determining environmental factors contributing significantly to the HGC condition of human populations in selected regions of the Venezuelan Andean range where HGC-related deaths are unusually high relative to the rest of the country [22,23]. One of every three cancer-caused deaths are due to HGC in males and one in four among females in the Andean area whereas it is the fifth cause of cancer fatalities in the rest of the country.

Among other potential causes, the transmission of bracken fern (*Pteridium* spp) terpenoid carcinogens, chiefly ptaquiloside, through the food chain [24-26] became suspects as ptaquiloside has been shown to impact severely animal health in various ways including induction of malignancies in urinary bladder in bovines, in addition to lung, ileum and mammary carcinomas in laboratory rodents [27,28]. Direct evidence in humans is lacking but indirect supporting evidence emerges from the distinct geographical distribution of HGC incidence in Meso and South America along the Andean range [22,23,29]. HGC incidence and mortality are invariably associated with geographical elevation: HGC records in highlands are systematically higher (where Pteridium ferns grow abundantly) than in lowlands (where this plant is rare or absent). The exception, Chile, (where Pteridium ferns do not occur) registers the opposite trend as HGC incidence is higher among people living on the coast and distinctly lower in Andean townships.

The increasing evidence in favor of a Hp(-)-Pteridium-HGC connection [30,31], led us to propose recently a hypothetical mechanisms whereby Hp and bracken ptaquiloside may be complimentary factors in the development of HGC in bracken exposed populations [32]. This notion might be supported further by a higher resolution study of Hp prevalence among people living in two adjacent regions with contrasting HGC mortality rates and vegetational composition as far as bracken fern invasion of pastures and water catchments. For this study we selected districts of south Maracaibo lake basin as the lowland representative and the adjacent highlands of the Andean range of Mérida State of Venezuela. To this end we undertook a *H. pylori* infection prevalence study as detected by the serum Immunoglobulin G (IgG) response, a recommended procedure for this kind of survey [33]. The study encompassed a total 1202 gastro-asymptomatic healthy adults. The data was then contrasted against the HGC death rates at the district level.

Materials and Methods

Ethical approval

The study was conducted under the ethics code of the Corporación de Salud del Estado Mérida, dependent on the Ministerio de Salud y Desarrollo Social of Venezuela, which conform the principles of the Declaration of Helsinki.

Experimental design

Cross-sectional survey of *H. pylori* seroprevalence status determined by an in house Enzyme Linked Immunosorbent Assay (ELISA) of IgG antibodies developed from four local bacterial strains. *H. pylori* assays were performed in matched populations according to sex and age group, from ten districts of western Venezuela comprising Mérida and Zulia States.

Study population

All participants were informed of the study and joined in voluntarily. All of them were enrolled during their visit to local public hospitals while requesting an official health certificate but not medical care, all declaring no relevant epigastric symptoms (acid reflux, local pain, recurrent indigestion). A total of 602 outpatients from the Maracaibo Lake area [360 females (59.8%) and 242 males (40.2%)] and 600 subjects from the Mérida mountainous region [452 females (75.33%) and 148 males (24.7%)] aged 15-65 years met all the selection criteria: in addition to information about age, sex and residential address within the districts under study, volunteers were selected only if fasting at the time of blood collection, over 10 years of residence in the area, no previous medical records of gastric pathologies, no antibiotic treatment received in the previous 6 months, and never treated for *H. pylori* eradication. The socioeconomic status and educational level was generally low (income level d, primary school exposure). Vein blood samples (10 mL/patient) were collected and immediately centrifuged at 3000 G to separate cell content. Sera were frozen (-20 °C) until analysis of *H. pylori* antibody levels.

Laboratory procedures

Enzyme-linked immunosorbent assay of IgG antibody levels: Sonicated crude extracts of four *H. pylori* strains, obtained from clinical antral biopsies from Hp(+) confirmed patients of Hospital Universitario de los Andes HULA in Mérida, were prepared. Ninety six flat bottom microwell plates (Sigma-Aldrich, St. Louis, Missouri, USA) were coated with 100 µL of 2 g/mL *H. pylori* extract in carbonate-bicarbonate buffer solution (0.05 M pH 9.5) at 1:125 dilution and were incubated overnight at 6 °C. Plates were washed five times with wash solution (PBS 0.1 M, pH 7.4, Tween 20 0.5%). All wells were filled with 100 µL of blocking solution (PBS-bovine albumin 1%-Tween 20) for 90 minutes prior to analysis. Subsequently plates were washed five times with wash solution. Serum samples (100 µL) were added in duplicate and incubated at 22 °C for 90

Table 1: Human population of surveyed districts, subject distribution and *Helicobacter pylori* IgG seroprevalence in two geographically distinct, vicinal regions of western Venezuela in the lowlands of the Maracaibo lake basin and the highlands of the Andean Mérida State.

			Hp(+) IgG prevalence ($CI_{95\%}$)[a]		
Area	**Population of surveyed districts**	**Subjects (males/ females)**	**% Males**	**% Females**	**% Total**
Lowland (Zulia)	142.29	602 (242/360)	92.4 (85.6-99.3)	79.8 (62.6-97.1)	85.0 (72.7-97.3)
Highland (Mérida)	101.645	600 (148/452)	93.8 (90.4-97.7)	81.7 (76.3-87.1)	87.2 (83.2-92.3)

[a]Hp(+): *Helicobacter pylori* positive individuals; IgG: Immunoglobin G.

min and wells were drained. After an additional washing with wash solution 100 µL of *H. pylori* IgG antibody conjugate at 1:20,000 v/v dilution was added to each well and was incubated for 60 min at 22 °C in the dark. The enzymatic reaction was quenched with Stop solution (H_2SO_4 2.5 N). Spectral absorbances were determined within 15 minutes after quenching.

Outcome variable: The activity of specific antibodies against the mixed *H. pylori* extract was estimated by the ELISA photospectral response Absorbance (Abs) at 405 nm using a Multiskan Plus V 2.01 Microplate photometer (Thermo Fisher Scientific, Houston Texas, USA). An antibody titer cutoff of Abs 0.410 au was established for the Hp(+) cases (median = 0.447, sd = 0.029; min = 0.400; max 0.498) and all values below 0.287 au were taken as Hp(-) people who showed a median of 0.266 (sd = 0.024; min = 0.2; max = 0.292), normal distribution and statistically differentiated ($p < 0.001$). Neonatal sera of children from Hp free mothers obtained from the nursery ward of Hospital de la Universidad de Los Andes, Mérida, were used as negative controls. Positive control sera were obtained from Hp infected patients confirmed by the unease test and positive pathological observation of *H. pylori* in endoscopies kindly provided by Instituto Dr. Luis E Anderson, Centro de Control de Cancer Gastrointestinal, San Cristóbal, Táchira, Venezuela.

Gastric cancer death rates: Age standardized mortality rates were calculated from crude rates obtained from Corporación de Salud del Estado Mérida (CSM) and Estado Zulia (CSZ) in the 1986-2006 period, using the Venezuelan population as reference. We gained access to mandatory gastric cancer death medical records of all reported individuals in specific townships covering the 1996-1999 periods. This data was used to estimate death rates in areas with 10,000 inhabitants or less in sanitary districts of the mountain region from which blood samples of healthy subjects were extracted for the Hp IgG seroprevalence study. Death record rather than gastric cancer incidence was utilized because of the elevated proportion of deaths due to this condition, the late HGC detection in the general population and the absence of HGC prevention programs.

Statistics: Cutoff spectral absorbances were estimated by fitting Hp(-) and Hp(+) cases separately into logistic curves ($\chi^2 \leq 7 \times 10^{-4}$). The mean of the 0.95 percentile upper group in the Hp(-) cases and the mean of the lowest 0.95 percentile in the Hp(+) cases were taken as cutoff values.

Comparison of the means in serum IgG antibody response was estimated using one way ANOVA at $p < 0.05$ for statistical significance. Other comparisons were executed using the non-parametric Kruskal-Wallis method at $p < 0.05$ using the χ^2 approximation, for statistical significance. Calculations were performed with Statistix V 7.0 (Analytical Software Inc. Tallahase, Florida, USA) and logistic curve analysis with Microcal Origin V 5 (Microcal Software Inc., Northampton, Massachussets, USA).

Results

H. pylori general status

IgG titers in *H. pylori* negative [Hp(-)] and positive groups [Hp(+)] were separated satisfactorily by the in-house immunoassay ($p < 0.001$, F = 620.6). The mean difference between the top quartile of Hp(-) and bottom quartile of Hp(+) Abs responses was also statistically significant ($p < 0.0001$, F = 2217). Therefore, interference or modulation of the immune response by other bacteria was disregarded.

Table 1 collects the general geographical and sex distribution of the 1202 volunteers. There were more female (812) than male (390) patients in both locations. Subjects in the 25-44 years age band constituted 55% of the sample in both sexes. Broadly speaking, Hp(+) seroprevalence was generally high in both lowland (Zulia State) and highland (Mérida State) populations. While consideration of all tested volunteers gave similar Hp(+) seroprevalence figures in both regions, a greater proportion of males appeared infected than females but the difference was not statistically significant after age adjustment for males ($p = 0.734$) or females ($p = 0.733$). The Relative Risk (RR) of *H. pylori* infection of males as compared with females in the lowlands was only 1.17 ($CI_{95\%}$ 0.99-1.36), a figure not distinguishable from the highland locations [RR: 1.16 ($CI_{95\%}$ 1.06-1.25) ($p = 0.845$)] considering all age groups.

When subjects were discriminated by group age from 15 to 64 years (group A: 15-24 years; B: 25-44 years; C: 45-64 years), the age-dependent Hp(+) seroprevalence showed moderate variation (Table 2). Females showed a slight increase of Hp IgG response with age in the lower regions but not in the mountain zone. Also female prevalence was statistically distinct relative to males in age groups A and B and became equal in group C. Males generally became Hp infected at an earlier stage with prevalence rates over 90% in both areas. There was a tendency towards a lower rate with age progress in males probably due to age-related infectious recession or lowered IgG titers as a result of declining immune response in the elderly [34]. However, values were not statistically differentiated.

As a whole, pondered mean age of cases [Age × Nº Hp(+)/Age × Nº(Hp(-) + Hp(+))] in the sample was 29.2 years for males and 26.4 years for females in the lowlands and 37.3 years for males and 29.9 years for females in the highlands, which may suggest an earlier acquisition of *H. pylori* in the lowlands. It has been shown that *H. pylori* infection occurs early in childhood with adult acquisition being much less common [35,36].

H. pylori prevalence and cancer rates by location

Knowing the long term (10 years) residence location of patients at specific districts, it made sense to match *H. pylori* prevalence data with gastric cancer death rates in these locations. Table 2 collects the results where the city of Cabimas (pop. 265,202 hab. 1999) was taken as representative of the lowland Zulia State whose population and GC rates were used as standards. Additionally, El Vigía, while also located in the lowland Maracaibo lake basin, occupied the transition territory along the Andean piedmont. For decades the placement of this town has favored economic and familial relationships with the mountain zone to the south. The rest of districts in Table 2 correspond to valleys and uplands in the Andean hinterland of Mérida State. HGC Odds Ratios (OR) were calculated against Zulia's population as standard and the HGC rates observed there in the period 1996-1999 to match the individual records obtained for smaller Andean districts. A clear trend towards much larger HGC-related death rates appears. Also, HGC rates at El Vigía reflect its area of influence on the nearby mountains in the same district where many patients suffering from gastric malignancy come from.

Table 2: Variation of *Helicobacter pylori* IgG seroprevalence with age group in the lowland and highland regions of western Venezuela comprising states Zulia and Mérida, respectively. Data for 1202 volunteers in all the sanitary districts (10) surveyed in this study. Average ± SE (N = 5 per region), [$CI_{95\%}$].

		Age group[a,x]		
Region	**Gender**	**15-24**	**25-44**	**45-64**
Lowlands	Male	94.2 ± 4.32[a,x] [80.6-107.6]	92.0 ± 0.5[a,x] [90.6-93.5]	84.6 ± 11.85[a,x] [46.85-122.9]
	Female	73.2 ± 8.4[b,x] [46.4-100.0]	75.0 ± 3.4[b,x] [64.2-85.9]	88.4 ± 7.5[a,x] [64.9-112.3]
Highlands	Male	90.0 ± 8.2[a,x] [69.0-111.0]	90.1 ± 4.1[a,x] [79.6-100.7]	78.7 ± 7.37[a,x] [59.8-97.7]
	Female	76.7 ± 5.6[a,x] [62.2-91.1]	74.5 ± 2.9[b,x] [66.9-82.1]	78.9 ± 5.9[a,x] [65.9-95.9]

[a]Equal letters indicate no statistical difference ($p > 0.05$) between male/female Hp(+) prevalence within the same age group in the same region. Hp(+): *Helicobacter pylori* positives; [x]Equal letters indicate no statistical difference ($p > 0.05$) between Hp(+) prevalence of the same gender and age group across regions.

Frequency of bracken growth sightings in surveyed districts

For many years we have studied several populations of bracken ferns in the Andean States of Venezuela and the carcinogenic compounds found there [22,23,31,37]. During the development of the current study we recorded the frequency of bracken growth sightings in the districts surveyed for *H. pylori* prevalence as dense growth on road edges, cattle paddock margins and as thickets inside pastureland supporting cattle. The bracken growth frequency was recorded in four categories depending on the ratio of observed positives for all visits: absent, occasional to 20%, 21-50% and above 50%. Results are collected in Table 3 which shows a distinct preference of bracken for mesothermic intermediate altitudes in humid mountain slopes and valleys, whereas megathermic lowlands, however humid, or high elevations near the night frost line did not sustain bracken except in occasional spots.

Discussion

Hp IgG seroprevalence

The results of our study reveal generally elevated Hp(+) seroprevalence rates of both sexes and ages above 15 years, in consonance with similar studies in most developing countries. Promiscuity, deficient sanitation and inadequate sewage disposal in the humid megathermic lowland districts may facilitate the spread of the infection as well as poverty and squatter housing in mesothermic and cold mountain areas [38,39]. While Hp(+) seroprevalence in asymptomatic volunteers was greater among males than females, which is in agreement with most published reports, there was no difference in the relative risk of *H. pylori* infection among males or females in the two zones (Table 1). Known Hp transmission avenues include food and water which have been contaminated in the household, saliva exchange through mouth contact among adults and children and others on the general fecal-oral and upsurge of stomachic fluids

Table 3: *Helicobacter pylori* IgG seroprevalence in the 15-64 y age range and gastric cancer death rates by sex, and bracken occurrence in or around pasturelands in selected districts of western Venezuela comprising a 0-3000 m elevation gradient.

	Hp IgG prevalence(%)[a]		GC odds ratio ($CL_{95\%}$)[b]		
Location (elevation m)	**Males**	**Females**	**Males**	**Females**	**Frequency of bracken[c]**
Cabimas (10 m)	94.6	82.4	1.0	1.0	none
El Vigía (110 m)	86.2	64.7	4.6 (2.0-7.3)	3.2 (0.4-6.8)	x
Santa Cruz (590 m)	74.1	80.8	5.4 (2.3-8.5)	6.3 (5.8-7.8)	xx
Tovar (950 m)	76.0	84.7	7.8 (5.5-10.2)	8.0 (5.0-11.2)	xxx
Bailadores (1720)	90.3	70.7	8.5 (2.8-14.0)	5.7 (4.1-10.5)	xxx
Mucuchíes (2950)	92.3	72.9	4.1 (1.9-6.4)	5.01 (3.5-5.0)	x

[a]Sex standardized death rates due to gastric cancer was diagnosed by reporting physician in death certificate; [b]Odds ratios were calculated against Zulia State GC (gastric cancer) rates using the population of this Federal entity: 2,781,000 (1999) as standard; [c]Relative frequency of bracken sightings during surveys in the visited districts: x: occasional to 20%; xx: 21-50%; xxx: > 50% (See text for details).

gateways [38]. Risk of primary and secondary infection is thus increased by lack of adequate personal and household sanitation and sewage disposal, all of these common features of the study populations. The *H. pylori* infection prevalence values found here are consistent with an earlier endoscopic and histological diagnosis survey of geographically limited scope in the vicinal Andean Táchira State [14], which validates the immunoassay method here used.

Gastric cancer death rates and *H. pylori* prevalence

Results of Table 3 do not show any association between Hp IgG seroprevalence and HGC. Although this result is in agreement with the general consensus, there are still discrepancies. On the one hand, according to the meta analysis of Huang, et al. [39], the Relative Risk (RR) of HGC incidence in *H. pylori* infected patients compared with uninfected controls was 1.81 ($CI_{95\%}$ 1.16-2.84) for all case-control studies. Cohort surveys furnished a stronger association: RR 2.24 ($CI_{95\%}$ 1.15-4.40) with confirming reports appearing thereafter [40-42].

On the other hand, the predictive value of *H. pylori* infection for future HGC development in asymptomatic people, the conceptual framework in which our study is positioned, is less clear. People in large communities and entire countries such as India, Bangladesh, and several African countries possess elevated levels of Hp(+) seroprevalence but low gastric cancer incidence rates [43]. Similarly, remote aborigine communities in Australia and South America where HGC is virtually unknown show very high Hp(+) seroprevalence rates (97.9%) among adults [5,15,43-47]. Other sources report a lack of association between Hp(+) seroprevalence and gastric pathologies in isolated communities of Nepal despite *H. pylori* regional differences [48]. Our results contribute to support this view. By contrast, other studies do find such associations, *e.g.* in southern Colombia [38] and in some Chinese provinces, Changle, Hong Kong, and Shandong [49,50]. Nonetheless, other parts of China [51] and Italy [52] do not show the same pattern.

HGC incidence in *H. pylori* patients has been attributed to a differential distribution of bacterial genotypes of varying virulence, including *iceA* gene, cytotoxin-associated gene *cagA* and vacuolating cytotoxin-associated gene *vacA* [42,50,53]. Statistical correlations vary from weak to strong in a few other reports [43,54-58]. As opposed to this, prospective studies do not show a clear correlation of $cagA^+$ Hp prevalence among asymptomatic patients living in high risk HGC regions when compared with lower risk zones in developing countries [59,60]. Still other investigations do not report associations between *iceA, cagA* or *vacA* status or their combination and the gastric clinical condition of patients in widely different countries as Japan, Korea, the United States and Colombia [61]. In the Venezuelan case, an earlier survey in Mérida State where our high HGC risk population resides, gave 50% $cagA^+$ prevalence [18], a low figure relative to other communities in this country (86%) where HGC death rates are much lower, according to these authors. Therefore, at the epidemiological level the frequency of these genotypes as a cause of non-cardia HGC remain to be demonstrated.

We also observed that there was a gradient of HGC death rates as elevation of surveyed settlements increased. Contrasts of HGC incidence rates between low and high areas are on record but to the best of our knowledge there seems to be a paucity of data regarding the HGC death rate change with an elevational gradient including several intermediate data points such as we report here.

Because altitude by itself is an unlikely contributor, other environmental stresses related to geographical elevation must be responsible. Among the most notorious in our geographical area, and possibly in other tropical countries with humid mid altitude mountains around the world as well, the increasing enrichment of the low vegetation cover by invading bracken fern (*Pteridium* spp.) in the megathermic to mesothermic gradient related to elevation that we have observed consistently in the study area [22,23,37] is a risk factor to consider seriously. Bracken is only rare at low elevation in the neotropics

but acquires the status of invading weed above 600 m and up to 2400 m [37], just where the Andean districts with the highest HGC rates are located. The district of Mucuchíes/2950 m), which deviates from this tendency, is above the ecological range of bracken. Yet, it is still within the trade route of unprocessed dairy products from lower bracken infested zones. Other environmental HGC propensity factors cannot be ruled out.

As *Pteridium* ferns invade pasturelands of small dairy cattle homesteads, dairy products and meat from these animals consumed locally by small communities and water runoff from bracken thickets are exposed to higher concentrations of the bracken carcinogen ptaquiloside for extended periods [24-26,62]. This compound is a powerful DNA alkylating agent causing specific mutations of oncogenes [63,64], an effect not yet recorded for Hp mutagenesis [65]. Convergence of circumstances, Hp insult and dietary mutagens such as ptaquiloside may constitute synergic effects leading to gastric neoplastic anomalies.

It can be concluded that Hp IgG seroprevalence is widespread among the communities studied in western Venezuela with values comparable to other developing countries and is not a predictor of HGC in these populations. Other propensity cofactors are required among which dietary intake of animal products laced with bracken carcinogens cannot be disregarded as an environmental-dietary risk factor.

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