

Multiple infections by EBV, HCMV and *Helicobacter pylori* are highly frequent in patients with chronic gastritis and gastric cancer from Southwest Mexico

An observational study

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Abstract

The chronic inflammation and damage to the gastric epithelium induced by *Helicobacter pylori* (*H. pylori*) are the main risk factors for gastric cancer development. Epstein-Barr virus (EBV) and human cytomegalovirus (HCMV) induce chronic inflammation and have been found in gastric tumors. The objectives this observational study were to determine the frequency of multiple infections by *Helicobacter pylori*, Epstein-Barr virus (EBV) and human cytomegalovirus (HCMV) and to relate the infection by EBV and HCMV with *H. pylori vacA/cagA* genotypes in patients with chronic gastritis or gastric cancer. DNA from *H. pylori*, EBV and HCMV was detected by PCR in biopsies from 106 Mexican patients with chronic gastritis and 32 from gastric cancer. The *cagA* status and the *vacA* genotypes of *H. pylori* were determined by PCR. In chronic gastritis and gastric cancer EBV was found in 69.8% and 87.5%, HCMV in 52.8% and 53.1%, and *H. pylori* in 48.1% and 40.6%, respectively. In chronic gastritis, 53% of *H. pylori*⁺ patients were EBV⁺ and 33% were both EBV⁺/HCMV⁺; in gastric cancer, 92.3% of *H. pylori*-infected individuals were EBV⁺ and 46.1% were EVB⁺/HCMV⁺. All the intestinal- and mixed-type tumors and the 83.3% of diffuse-type tumors were EBV⁺. No significant differences were found between single infections or coinfections with the diagnosis or the cancer type. The *H. pylori* genotypes were not related to EBV or HCMV infection. The frequency of dual infections by *H. pylori*, EBV and HCMV is higher in patients from southwest Mexico than other populations. It is likely that these pathogens act synergistically to induce inflammation and gastric cancer.

Abbreviations: CagA = cytotoxin-associated gene A, EBNA = nuclear antigen, EBV = Epstein Barr virus, HCMV = human cytomegalovirus, HDGC = hereditary diffuse gastric cancer, IARC = International Agency for Research on Cancer, LMP = latent membrane protein, NH₂Cl = monochloramine, SD = Standard deviation, VacA = vacuolating cytotoxin.

Keywords: chronic gastritis and gastric cancer, epstein-barr virus, *Helicobacter pylori*, human cytomegalovirus

1. Introduction

In 2013, gastric cancer was ranked third in incidence and mortality in developing countries.^[1,2] In the United States of America, the gastric cancer incidence rate on Hispanic men was

73% higher than for non-Hispanic white men; among the Hispanic woman, this rate was more than twice than that among non-Hispanic white woman.^[3] Based on Lauren's classification, the most common histologic types of gastric adenocarcinoma are

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the intestinal-type and the diffuse-type, which differ in the etiology and carcinogenic pathways.^[4] Intestinal-type gastric cancer initiates inflammatory changes and focal necrosis that evolve into chronic gastritis, atrophic chronic gastritis, intestinal metaplasia, dysplasia and cancer.^[5,6] On the other hand, the histologic changes that occur during diffuse carcinoma progression are still unknown, which starts with gastritis throughout the stomach but is not atrophic.^[7]

Gastric cancer has a recognized infectious etiology. Chronic infection with *Helicobacter pylori* (*H. pylori*) is the risk factor that contributes the most to gastric carcinogenesis, although only 5% of infected individuals develops the disease.^[3] *H. pylori* can induce gastric carcinogenesis by 2 main mechanisms:

1. by indirect effects, stimulating a local chronic inflammatory response and
2. by direct effects on the molecular composition of epithelial gastric cells through the toxic action of its virulence factors.^[8]

H. pylori infection stimulates a chronic inflammatory response that leads to an increased cellular turnover that may result in the accumulation of mitotic errors. Moreover, persistent inflammation leads to atrophic gastritis, which is accompanied by hypochlorhydria or achlorhydria and the rise of the gastric pH. The clinical result depends on the severity and distribution of gastritis as well as deficiency in gastric acid secretion.^[9] Through the virulence factors CagA and VacA, *H. pylori* directly promotes mutations in cell cycle regulatory genes, deficiencies in DNA repair mechanisms, loss of cell adhesiveness and epigenetic changes that can alter cell function and promote cell autonomy and malignant transformation.^[8,10] Only a portion of the *H. pylori* strains possess the *cagA* gene but all encode the *vacA* gene. The latter is a polymorphic gene and the strains encoding the allelic combination *s1m1* are associated with pre-neoplastic gastric lesions and cancer. The majority of the *cagA*⁺ strains are *vacA s1m1*.^[11]

Epstein-Barr virus (EBV) can infect the superficial epithelium of the stomach through B cells carrying reactivated EBV, which may initiate carcinogenesis.^[12,13] The EBV was classified as a type I carcinogenic by the International Agency for Research on Cancer (IARC) and growing evidence associates EBV with gastric cancer.^[14,15] Results from in vitro studies suggest that B cells infected with EBV generate a high rate of infectiousness on epithelial cells.^[16] EBV can induce gastric epithelial cell death or persist as a latent infection and promote cancer progression.^[17] Some gastric carcinomas harbor the EBV monoclonal genome in each cancer cell; this finding suggests that all those cells originated from the same infected progenitor cell. The viral monoclonality in EBV-positive gastric cancer strengthens the causal relationship between EBV and gastric carcinogenesis.^[18–20] Additionally, EBV-positive gastric cancer samples are characterized by recurrent mutations of *PIK3CA*, DNA hypermethylation and JAK2, CD274 and PDCD1LG2 amplification, which may be mechanisms involved in carcinogenesis.^[17,20] It is likely that EBV exerts its oncogenic effects through nuclear antigens (EBNAs) and latent membrane proteins (LMPs), that interact with signaling pathways and tumor suppressor genes.^[15] Based on the molecular characteristics that differentiate gastric tumors, the Cancer Genome Atlas Investigation Network defined EBV-associated gastric cancer as a specific subtype.^[21]

In adulthood, approximately 50% of the population is infected with *H. pylori* and up to 95% has EBV in a latent status. Both pathogens are acquired during childhood.^[16] The frequency and distribution of gastric cancer subtypes and the frequency of

H. pylori and EBV differ between geographical regions of the world.^[21] The EBV percentage of infection in gastric cancer varies between 4.3% and 18%.^[19] The EBNA-1 protein from EBV has not been detected in healthy subjects, however, it has been found in 4.1% to 37.3% of patients with non-ulcerous gastric disease, in 16.7% to 75.6% of individuals with ulcerous peptic disease and in 80% to 90% of gastric cancer cases.^[20]

Human cytomegalovirus (HCMV) is a herpesvirus that causes serious intrauterine infections as well as acute and chronic complications in immunocompromised patients.^[22] HCMV is not considered oncogenic, in spite of in vitro evidence that suggests that it can cause fibroblast transformation in culture.^[23] HCMV encodes approximately 185 genes from which UL133, UL135, and UL136 have been associated with gastric cancer. The UL33 expression rate in cancerous tissue is significantly higher than in normal tissue^[24] and the number of gastrointestinal disease cases caused by HCMV has increased, between them are ulcerative colitis and esophageal ulcers.^[25] There are still few studies documenting the frequency of HCMV infection in gastric diseases and it is still necessary to clarify the relationship of this virus with gastric cancer.

Gastric cancer incidence remains highly common in Mexico, Central and South America, and Asia but has substantially declined in high-income countries. Meanwhile, *H. pylori* prevalence is higher in low-income countries and among individuals with low socioeconomic status.^[26] There are few studies on the presence of EBV in digestive pathologies and it is likely that the importance of this virus in gastric carcinogenesis is being underestimated. EBV infection in gastric mucosa of patients with chronic gastritis and gastric cancer is a practically unexplored field in Mexico and only 2 studies have addressed EBV and *H. pylori* coinfection through quantification of specific antibodies.^[27,28] In this study, we estimated the frequency of single infections and coinfections by EBV, HCMV and *H. pylori* in patients with chronic gastritis or gastric cancer. In addition, we determined the prevalence of *H. pylori vacA/cagA* genotypes and evaluated the relation between the viruses and *H. pylori*-specific genotypes. Lastly, we analyzed the frequency of each pathogen on dual and triple infections by cancer type.

2. Materials and methods

2.1. Patients

A cross-sectional study was carried out in 138 patients, consecutively selected among those presenting symptoms of dyspepsia or presumptive diagnosis of gastric cancer. One hundred six underwent upper gastrointestinal endoscopy in the Specialized Unit in Gastroenterology and Endoscopy in Chilpancingo, Guerrero, Mexico. Thirty-two subjects were subjected to an endoscopic study to confirm gastric cancer in the Cancerology State Institute from Acapulco, Guerrero, Mexico. Patients without antimicrobial treatment and without consumption of proton pump inhibitors or gastric pH neutralizers during the 2 weeks prior to the endoscopic study were included. Patients under non-steroidal anti-inflammatory treatment or treated with immunosuppressors were excluded. Selection of patients was carried out from March 2012 to May 2015. The patients or their parents signed a written informed consent form and they were treated in accordance with the tenets of the *Declaration of Helsinki*. The study was approved by the Bioethics committee from from Autonomous University of Guerrero and by the Investigation Department of Cancerology State Institute.

2.2. Tissue obtainment and histological study

The endoscopic study was carried out after 1 night of fasting with a video processor and video gastroscope (Fujinon, Wayne, NJ). Two biopsies of antrum or body were taken from chronic gastritis patients and 2 tumor biopsies were taken from gastric cancer patients. A biopsy from each site was immediately fixed in 10% formalin for histological examination and another was placed in a buffer solution (Tris 10 mM pH 8.0, EDTA 20 mM pH 8.0, SDS 0.5%) in order to obtain total DNA for *H. pylori*, EBV and HCMV diagnosis. Biopsies for *H. pylori*, EBV and HCMV detection were maintained at -20°C until processing and formalin-fixed biopsies were embedded in paraffin. Four-micrometer sections of paraffin-embedded tissue were stained with hematoxylin-eosin for the histological study. The histopathological diagnosis was carried out according to the updated Sydney system^[29] or based on the Padova international classification for gastric dysplasia.^[30] Endoscopic and histopathological findings were only used to establish the diagnosis of the patients.

2.3. *H. pylori* detection and *vacA* genotyping

Total DNA from gastric biopsies was extracted by the Phenol-Chloroform method, after digestion with Proteinase K.^[31] *H. pylori* DNA was detected using the primers HPF 5'-GCT AAG AGA TCA GCC TAT GTC C-3' and HPR 5'-CAA TCA GCG TCA GTA ATG TTC-3', modified from those used by Chang et al, which amplify a fragment of the *rRNA 16S* gene.^[32] DNA integrity was verified adding a set of primers specific for *IL-1B* gene in the PCR for *H. pylori 16S rRNA* gene. *IL-1B* primers sequences were sense 5'-CAT TTG TCA GGT TCT TGA TC-3' and antisense 5'-GAA GTT TAG TCT TCC CAC TT-3' which amplified a 305 bp fragment. The signal and middle regions of *vacA* were genotyped by PCR, using the oligonucleotides previously used by Koehler et al^[33] and following the methodology described by Román-Román et al.^[34]

2.4. *cagA* detection

cagA detection was performed in *H. pylori rRNA 16S* positive samples by PCR, using the primers *cagAF* 5'-ATA ATG CTA AAT TAG ACA ACT TGA GCG A-3' and *cagAR* 5'-TTA GAA TAA TCA ACA AAC ATC ACG CCA T-3',^[35] which amplify a 297 bp fragment from constant region. In addition, a second set of primers was used in order to amplify a 500 to 850 bp fragment from the 3' *cagA* variable region *cag2F* 5'-GGA ACC CTA GTC GGT AAT G-3' and *cag4R* 5'-ATC TTT GAG CTT GTC TAT CG-3'. PCR reactions were carried out in a total volume of 25 μL containing 1.7 mM MgCl_2 , 0.2 mM dNTP's (Invitrogen, Carlsbad, CA), 5 pmol from each primer, 1 U Platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, CA) and 300 ng total DNA. Cycling conditions were as follows: 1 cycle at 94°C for 5 min, 30 cycles at 94°C for 40 s, 56°C for 30 s and 72°C for 50 s, with a final extension cycle at 72°C for 10 min. The resulting PCR products were resolved in 1.5% agarose gels, stained with ethidium bromide and observed under ultraviolet light (UV). Samples were considered *cagA*-positive when either one or both PCR products were observed.

In all PCR reactions, DNA from strain ATCC 43504 *H. pylori vacA s1m1/cagA+/babA2+* was used as a positive control and the template DNA was substituted with sterile deionized water in the negative control. Total DNA from a *vacA s2/m2* positive gastric biopsy was used as a positive control for *s2* and *m2* allele

genotyping. All PCR reactions were run in a Mastercycler EP gradient thermocycler (Eppendorf, Hamburg, Germany).

2.5. EBV and HCMV detection

DNA from EBV was identified using the set of primers EBNA1-F 5'-AGG CCA TTT TTC CAC CCT G-3' and EBNA1-R 5'-CTT CTC CTG GGT CAT CTG CG-3', which amplify a fragment of 114 bp from the *EBNA1* gene. Detection of HCMV DNA was performed using the set of primers forward UL44-F 5'-GTG CGC TCA AGG AGA ACA C-3' and reverse UL44-R 5'-TGC ACG TAG AAC TTG GTC AG-3', that amplify a 181 bp region from the *UL44* gene. PCR reactions were performed in a total volume of 20 μL containing 200 ng DNA, 4 mM MgCl_2 , 0.2 mM dNTP's, 5 pmol of each primer for EBNA1, 0.4 μM of each primer for UL44 and 1 U Platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, CA). All PCR reactions were run in a Mastercycler EP gradient thermocycler (Eppendorf, Hamburg, Germany). Cycling conditions included 1 cycle of initial denaturation at 94°C for 5 min, followed by 40 cycles of 94°C for 20 s, 57°C for 20 s for *EBNA1* gene (58°C for UL44), 72°C for 30 s and a final extension cycle at 72°C for 7 min. All PCR products were resolved in 2.5% agarose gels, stained with ethidium bromide and visualized under UV light. Samples were considered positive for EBV or HCMV when the PCR products of the corresponding sizes were identified. As a positive control, an EBV+/HCMV+ sample was used. The specificity of viral DNA in the sample was verified by sequencing.

2.6. Statistical analysis

The frequencies of qualitative variables and the mean \pm standard deviation (SD) of parametric quantitative variables were determined. The X^2 test or the Fisher exact test were used for frequency comparisons between groups and Student's *t* test was used for mean comparisons. A *P* value of $<.05$ was considered statistically significant. All the statistical calculations were performed using Stata V.9.2 software (College Station, TX).

3. Results

3.1. Patients

From the 138 patients included in the study, 106 (76.8%) had histopathological diagnosis of chronic gastritis and 32 (23.2%) of gastric cancer, from which 7 (21.9%) were intestinal-type, 18 (56.2%) diffuse-type, 3 (9.4%) mixed-type and 4 (12.5%) had other types of cancer. The mean age was 50 ± 17 years (4–89 years) for chronic gastritis patients and 63 ± 13 years (37–85 years) for gastric cancer patients. In both groups, the female gender predominated, 55.8% of the total patients. Groups were significantly different in age ($P < .001$) and educational level ($P < .001$), Table 1.

3.2. EBV, HCMV and *H. pylori* prevalence

Of all patients, 73.9% (102/138) were EBV-positive, 52.9% (73/138) were HCMV-positive and 46.4% (64/138) were positive for *H. pylori*. EBV, HCMV and *H. pylori* prevalence varied according to diagnosis, Table 2. From *H. pylori*-positive cases, 89% (57/64) were *vacA s1m1* and 79.7% (51/64) were positive for *cagA*. No significant differences were found in *H. pylori* genotype distribution between groups, Table 2.

Table 1**Sociodemographic features of chronic gastritis and gastric cancer patients.**

	Chronic gastritis n=106	Gastric cancer n=32	P value
Age (mean ± SD; years)	50 ± 17	63 ± 13	<.001 [†]
Gender n (%)			
Female	60 (56.6)	17 (53.1)	.73 [€]
Male	46 (43.4)	15 (46.9)	
Education n (%)			
Bachelor or higher	50 (47.2)	2 (6.2)	<.001 [‡]
High school	18 (17)	4 (12.5)	
Middle school	9 (8.5)	2 (6.2)	
Elementary school	22 (20.7)	12 (37.5)	
No studies	7 (6.6)	12 (37.5)	

[†] Student's *t* test.[€] χ^2 test.[‡] Fisher exact test.

3.3. EBV, HCMV and *H. pylori* (*vacA/cagA*) coinfections

Of all patients, 41.3% (57/138) were EBV⁺/HCMV⁺, this coinfection reached 50% (16/32) in gastric cancer and 38.7% (41/106) in chronic gastritis. On the other hand, 28.3% (39/138) of patients were *H. pylori*⁺/EBV⁺. Prevalence of *H. pylori*⁺/EBV⁺ cases was higher in gastric cancer than chronic gastritis, 37.5% (12/32) and 25.5% (27/106), respectively. Of the total cases, 24% (33/138) were *H. pylori*⁺/HCMV⁺ and this coinfection was more frequent in the group with gastritis, 25.5% (27/106). Seventeen percent (23/138) of patients were *H. pylori*⁺/EBV⁺/HCMV⁺ and 3.6% (5/138) of patients were free from infection, Table 3. In chronic gastritis, 53% (27/51) of *H. pylori*⁺ cases were EBV⁺ and 33% (17/51) were EBV⁺/HCMV⁺. In gastric cancer,

Table 2**EBV, HCMV and *H. pylori vacA/cagA* genotype frequencies in patients with chronic gastritis and gastric cancer from Guerrero state, Mexico.**

	Diagnosis		P value
	Chronic gastritis n=106	Gastric cancer n=32	
EBV n (%)			
Negative	32 (30.2)	4 (12.5)	.07 [‡]
Positive	74 (69.8)	28 (87.5)	
HCMV n (%)			
Negative	50 (47.2)	15 (46.9)	.97 [€]
Positive	56 (52.8)	17 (53.1)	
<i>H. pylori</i> n (%)			
Negative	55 (51.9)	19 (59.4)	.46 [€]
Positive	51 (48.1)	13 (40.6)	
<i>H. pylori vacA</i> genotype			
<i>s2m2</i>	3 (5.8)	2 (15.4)	.64 [‡]
<i>s1m1</i>	45 (88.2)	11 (84.6)	
<i>s1m1/s1m2</i>	1 (2)	0	
<i>s1/m0</i> [*]	1 (2)	0	
<i>s0/m0</i> [*]	1 (2)	0	
Total	51 (100)	13 (100)	
<i>H. pylori cagA</i> status			
Negative	8 (15.7)	5 (38.5)	.07 [€]
Positive	43 (84.3)	8 (61.5)	
Total	51 (100)	13 (100)	

[€] χ^2 test.[‡] Fisher exact test.^{*} Undefined sample for *vacA m* or *s* regions.**Table 3*****H. pylori*, EBV and HCMV coinfection in patients with chronic gastritis and gastric cancer.**

<i>H. pylori</i> , EBV and HCMV	Diagnosis		P value [‡]
	Chronic gastritis n (%)	Gastric cancer n (%)	
<i>H. pylori</i> ⁻ /EBV ⁻ /HCMV ⁻	3 (2.8)	2 (6.2)	.21
<i>H. pylori</i> ⁻ /EBV ⁻ /HCMV ⁺	5 (4.7)	1 (3.1)	
<i>H. pylori</i> ⁻ /EBV ⁺ /HCMV ⁻	23 (21.7)	6 (18.8)	
<i>H. pylori</i> ⁻ /EBV ⁺ /HCMV ⁺	24 (22.6)	10 (31.2)	
<i>H. pylori</i> ⁺ /EBV ⁻ /HCMV ⁻	14 (13.2)	1 (3.1)	
<i>H. pylori</i> ⁺ /EBV ⁻ /HCMV ⁺	10 (9.4)	0	
<i>H. pylori</i> ⁺ /EBV ⁺ /HCMV ⁻	10 (9.4)	6 (18.8)	
<i>H. pylori</i> ⁺ /EBV ⁺ /HCMV ⁺	17 (16)	6 (18.8)	
Total	106 (100)	32 (100)	

[‡] Fisher exact test.

92.3% (12/13) of *H. pylori*-infected patients were EBV⁺ and 46.1% (6/13) were EBV⁺/HCMV⁺, Table 3. No differences were found in EBV and HCMV distribution according to the *H. pylori* genotypes between groups, Table 4.

3.4. EBV, HCMV and *H. pylori* infection by type of cancer

Among all cancer patients, 93.8% (30/32) were positive for at least one infectious agent. All intestinal- (7) and mixed-type (3) tumors were EBV⁺; in contrast, 83.3% (15/18) of diffuse-type tumors tested positive for EBV. HCMV frequency was 28.6% (2/7), 61.1% (11/18) and 100% (3/3) for intestinal-, diffuse- and mixed-type cancer, respectively. Ninety-one percent (29/32) of all tumors were EBV⁺, HCMV⁺ or EBV⁺/HCMV⁺, in 40.6% (13/32) of cases *H. pylori*, *H. pylori*/EBV or *H. pylori*/EBV/HCMV were found and 40% (12/30) of all infection cases were *H. pylori*⁺/EBV⁺. From the tumor-infected cases, 20% (6/30) were *H. pylori*⁺/EBV⁺/HCMV⁺. The 42.8% (3/7) of intestinal-type cancer and 27.7% (5/18) of diffuse-type cancer were *H. pylori*⁺/EBV⁺, Table 5. *H. pylori* infection was present in 42.8% (3/7) of intestinal-type cancer cases, in 33.3% (6/18) of diffuse-type cancer and in 66.6% (2/3) of mixed-type cancer cases.

4. Discussion

Twenty percent of malignant neoplasms are induced by infectious agents. *H. pylori* is a type I carcinogen for gastric cancer,^[36,37] nonetheless, about 50% of gastric tumors are *H. pylori*-negative. On the other hand, between 1.3% and 20.1% of gastric carcinomas contain EBV and the average of cases reaches 10% worldwide.^[38] Some tumors are associated with HCMV.^[24,39]

In this study, we analyzed EBV and HCMV frequency of infection in patients with chronic gastritis and gastric cancer with and without *H. pylori* infection and established the relation between the presence of a viral genome with the diagnosis of the patients and with *H. pylori* virulence factors. We found that the virus-*H. pylori* coinfections are more frequent in gastric cancer than chronic gastritis and that the EBV frequency of infection in gastritis and gastric cancer is higher than that reported in other populations.

In chronic gastritis patient's ages varied from 4 to 89 years. The youngest patients were 2 children of 4 and 6 years of age, EBV⁺ and EBV⁺/HCMV⁺, respectively. In gastric cancer, the minimum age was 37 years and the maximum age was 85 years. The

Table 4
EBV and HCMV infection and *vacA/cagA* genotypes in *H. pylori*-positive patients with chronic gastritis or gastric cancer.

<i>H. pylori</i> genotypes	Chronic gastritis		Gastric cancer	
	HCMV		HCMV	
	Negative n=24	Positive n=27	Negative n=7	Positive n=6
<i>vacA</i> n (%)				
<i>s2m2</i>	1 (4.2)	2 (7.4)	1 (14.3)	1 (16.7)
<i>s1m1</i>	20 (83.3)	25 (92.6)	6 (85.7)	5 (83.3)
<i>s1m1/s1m2</i>	1 (4.2)	0	0	0
<i>s1/m0*</i>	1 (4.2)	0	0	0
<i>s0/m0*</i>	1 (4.2)	0	0	0
<i>P</i> value [‡]	.44		> .99	
<i>cagA</i> n (%)				
Negative	5 (20.8)	3 (11.1)	3 (42.9)	2 (33.3)
Positive	19 (79.2)	24 (88.9)	4 (57.1)	4 (66.7)
<i>P</i> value [‡]	.45		> .99	

<i>H. pylori</i> genotypes	Chronic gastritis		Gastric cancer	
	EBV		EBV	
	Negative n=24	Positive n=27	Negative n=1	Positive n=12
<i>vacA</i> n (%)				
<i>s2m2</i>	2 (8.3)	1 (3.7)	0	2 (16.7)
<i>s1m1</i>	19 (79.1)	26 (96.3)	1 (100)	10 (83.3)
<i>s1m1/s1m2</i>	1 (4.2)	0	0	0
<i>s1/m0*</i>	1 (4.2)	0	0	0
<i>s0/m0*</i>	1 (4.2)	0	0	0
<i>P</i> value [‡]	.27		> .99	
<i>cagA</i> n (%)				
Negative	5 (20.8)	3 (11.1)	0	5 (41.7)
Positive	19 (79.2)	24 (88.9)	1 (100)	7 (58.3)
<i>P</i> value [‡]	.45		> .99	

[‡] Fisher exact test.
^{*} Undefined sample for *vacA* *m* or *s* regions.

youngest patient was diagnosed with diffuse-type gastric cancer and had a familial history of this tumor type. It is likely that this was a case of hereditary diffuse gastric cancer (HDGC), which constitutes 1% to 3% of gastric cancer cases.^[40,41]

Educational level is a parameter used to evaluate the socioeconomic aspect and the marginalization level in a population.^[42] In this research, 65% of gastric cancer subjects had no education or only had an elementary education. In 2016, Gómez-Dantés et al^[43] reported that Guerrero is one of the states in the Mexican Republic with the highest marginalization levels and that, among Guerrero people, gastric cancer is the third

cause of oncological mortality. The majority of patients included in this research come from hospitals that provide care for people with low socioeconomic status, who live in rural areas and do not have a social security program. This makes it difficult to access health services and favors gastric tumor late diagnosis, especially among people without schooling, lacking information and with scarce economic resources. In Mexico, as in most of the Latin American countries, the socioeconomic status of the population adds to the lack of a surveillance program and early detection of gastric cancer that results in high mortality from this cause.

Table 5
EBV, HCMV and *H. pylori* coinfection by cancer type.

<i>H. pylori</i> , EBV and CMV	Cancer type				Total n (%)
	Intestinal n (%)	Diffuse n (%)	Mixed n (%)	Others* n (%)	
<i>H. pylori</i> ⁻ /EBV ⁻ /HCMV ⁻	0	1 (50)	0	1 (50)	2 (100)
<i>H. pylori</i> ⁻ /EBV ⁻ /HCMV ⁺	0	1 (100)	0	0	1 (100)
<i>H. pylori</i> ⁻ /EBV ⁺ /HCMV ⁻	2 (33.3)	3 (50)	0	1 (16.7)	6 (100)
<i>H. pylori</i> ⁻ /EBV ⁺ /HCMV ⁺	2 (20)	7 (70)	1 (10)	0	10 (100)
<i>H. pylori</i> ⁺ /EBV ⁻ /HCMV ⁻	0	1 (100)	0	0	1 (100)
<i>H. pylori</i> ⁺ /EBV ⁺ /HCMV ⁻	3 (50)	2 (33.3)	0	1 (16.7)	6 (100)
<i>H. pylori</i> ⁺ /EBV ⁺ /HCMV ⁺	0	3 (50)	2 (33.3)	1 (16.7)	6 (100)
Total	7 (21.8)	18 (56.3)	3 (9.4)	4 (12.5)	32 (100)
<i>P</i> value [‡]	.57				

* Gastrointestinal stromal tumor; Cardia with infiltration due to poorly differentiated extensively ulcerated carcinoma; Lymphoproliferative process with acute, ulcerated inflammation suggestive of non-Hodgkin lymphoma.
[‡] Fisher exact test.

Several authors have detected EBV genome in epithelial cells from gastric tumors and this virus has been associated with carcinogenesis. However, most of these studies have focused on cancer samples and the frequency of viral infections (EBV and HCMV) associated with *H. pylori* infection in chronic gastritis is unknown. We found that EBV prevalence in chronic gastritis was 69.8% and 87.5% in cancer, higher than that found in the populations of Mexico City and southeast Mexico; in the previous studies, DNA from EBV was found in 8% and 10.67% of non-atrophic gastritis and tumor tissue, respectively.^[44] In children with non-atrophic gastritis from Mexico City, EBV seroprevalence was 64.3%.^[27] In adults from Mexico and Paraguay with the same type of gastritis, frequency of anti-EBV IgG antibodies was 92% and in gastric cancer cases was 94.7%.^[28] Ryan et al^[45] found DNA from EBV in 83%, 64% and 30% of gastric tissues from Honduran patients as well as in North American adults and children with gastritis, respectively. In gastric cancer patients, the frequency of EBV DNA varies from 7.9% in Asia to 50.2% in Turkey. In gastric cancer subjects from India, 90% tested positive for EBV EBNA1 gene.^[46] It has been observed that EBV frequency is geographically different.^[44] The discrepancies in EBV prevalence may be explained by differences in sensibility and specificity of the methods employed between the studies, by the diagnosis of the studied patients and by the population's geographical region of origin.

Chronic inflammation is a risk factor for cancer. HCMV induces chronic gastritis and is detected in a fraction of cancer cases, so it may be that this virus is etiologically related to gastric carcinogenesis as a single infection or in synergy with EBV or *H. pylori*. In this research, the HCMV prevalence in gastritis and gastric cancer was 52.8% and 53.1%, respectively. There are few data on HCMV prevalence in gastritis and stomach cancer and the relation of this virus with gastric carcinogenesis is not clear yet. In developed countries, 60% of the adult population is positive for anti-HCMV IgG antibodies and in developing countries, such as Mexico, this prevalence reaches 100%. A variable percentage of people remains free of infection and may acquire HCMV at any life stage. In most of the infected individuals, the virus remains in a latent form, with a probability of reactivation and infection with a distinct strain of HCMV.^[47] HCMV reactivation occurs in patients with immunosuppression of diverse etiology^[48,49] and in organ donors or recipients,^[50] among others. In 18 HIV-positive dyspeptic patients, Falasca et al^[51] found anti-HCMV IgG antibodies but in none of them, HCMV DNA was detected in antral biopsies. On the other hand, gastritis cases caused by HCMV have been reported in immunocompetent patients^[52] and gastric cancer cases that have evolved from HCMV gastritis. Interestingly, in some cases, treatment with antivirals has induced improvement in gastritis cases.^[53] To our knowledge, this is the first study that determines EBV and HCMV prevalence in gastric mucosa in Mexican patients with chronic gastritis and gastric cancer with and without *H. pylori* infection.

In chronic gastritis patients, *H. pylori* frequency was 48.1%, similar to that reported by Román-Román et al^[34] but smaller than to that informed by Paniagua et al^[54] and Martínez-Carrillo et al^[55] 40.6% of gastric cancer patients was *H. pylori*⁺, this prevalence is similar to that found in other studies in patients from Guerrero state.^[34,56] It is possible that frequency variation is influenced by patient information obtainment bias. In addition, it is not possible to rule out that patients had been received antimicrobial treatment for *H. pylori* in the recent past. It is likely that people have used antibiotics indicated for other infections.

Among the Mexican population, self-medication is a common practice, and although the sale of antibiotics without a medical prescription is prohibited in the country, Metronidazole is an antiparasitic drug that is part of the treatment scheme for *H. pylori*, which is an over-the-counter medication. Additionally, the probability of finding *H. pylori* in the biopsies decreases as structural and functional changes appear on the infected mucosa.

In patients with gastric pathology from Guerrero state, *H. pylori s1m1* and *cagA*⁺ strains predominate^[34] but does not explain gastritis and gastric cancer frequency. We found that in *H. pylori*⁺ patients, the *s1m1* genotype predominated with a frequency of 88.2% and 84.6% in chronic gastritis and gastric cancer, respectively. The frequency of *H. pylori cagA*⁺ was 84.3% in chronic gastritis and 61.5% in cancer. The *H. pylori s1m1/cagA*⁺ genotype is recognized as the most virulent one and increases gastric cancer risk. Our observations suggest that single and dual infections with EBV and HCMV contribute to gastritis and gastric cancer etiology.

We found that 41.3% of all patients were EBV⁺/HCMV⁺ and that 16.7% were *H. pylori*⁺/EBV⁺/HCMV⁺. The most frequent pathogen was EBV in both groups of patients. In chronic gastritis, the frequency of EBV⁺/*H. pylori*⁺ was 25.5% and in gastric cancer was 37.5%. The prevalence of EBV and EBV/*H. pylori* coinfection found in this study is lower than the seroprevalence of these agents in 525 patients over 30 years of age from Mexico and Paraguay with non-atrophic gastritis, atrophic gastritis, intestinal metaplasia and cancer.^[28] The differences in the age range and the diagnosis can partially determine the divergences in the prevalence of infections. The presence of EBV and *H. pylori* DNA is independent of the infection status and, although some EBV particles may have come from saliva, is very likely that the virus was found on infiltrating B cells and gastric epithelial cells. The detection method used prevents us from specifying the cellular source of the identified viral DNA. It has been proposed that EBV and *H. pylori* can act synergistically to cause and perpetuate gastritis.^[28]

EBV was found in 100% of intestinal- and mixed-type tumors and in 83.3% of diffuse-type tumors. We did not find significant differences in EBV frequency by cancer type, which is in agreement with that reported by other authors.^[15,28,44,46] Forty-three percent (3/7) of intestinal-type cancer cases and the 27.7% (5/18) of the diffuse-type were *H. pylori*⁺/EBV⁺; meanwhile, Shukla et al^[46] found this dual infection in higher proportions with 58.3% and 53.8% for intestinal- and diffuse-type cancer, respectively. These differences may be due to the populations studied and to the sensibility and specificity of the employed methods.^[57] The frequency of *H. pylori*⁺/EBV⁺ cases was higher in cancer (37.5%) than gastritis (25.5%). Our results support the hypothesis that both pathogens act in synergy to induce carcinogenesis, however, more studies addressing this matter are needed. *H. pylori* potentiates the inflammatory response in chronic gastritis and it drives gastric Th17 response through the HP0175 protein, a peptidyl prolyl *cis*, *trans*-isomerase (PPIase). It has been shown that HP0175 protein of *H. pylori* is able to elicit high production of IL-17 and IL-21 in gastric tumor infiltrating lymphocytes (TILs) and it promotes Th17 inflammation which, if prolonged and unabated, may be a condition that links the infection and gastric cancer.^[58] In double and triple infections, *H. pylori*, EBV and HCMV can act synergistically, enhancing the expression of IL-17 and maintaining an inflammatory state that more severely damages gastric mucosa.^[59-61] On the other hand, the oncoprotein CagA induces the secretion of IL-1 β , IFN- γ , and TNF- α . IL-1 β exerts its effects on gastric epithelial cells that

might lead to the activation or expression of oncogenes.^[58] It is known that *H. pylori* induces to monochloramine (NH₂Cl) generation and with it the EBV reactivation.^[62] Viral reactivation induces greater infiltration of B cells loaded with viral particles which increases the probability of epithelial cell infection.^[45,46,62,63] Dual infection increases IL-1 β , TNF- α and IL-8 production, augments immune cell infiltration and there is more damage to gastric mucosa by the effect of the infection and of a more intense chronic inflammation.^[16,45,59] It has been proposed that atrophic chronic gastritis and intestinal metaplasia caused by *H. pylori* augments gastric epithelium susceptibility to EBV infection and that in turn, viral infection promotes carcinogenesis.^[58] Meanwhile, CagA induces PLC γ activation, which favors EBV reactivation on infected B cells in vitro^[64] and both agents activate the β -catenin/TCF-4 pathway, which are transforming factors in gastric cells.^[16,27]

In conclusion, our results indicate that the frequency of dual and triple infections by *H. pylori*, EBV and HCMV — which are 3 infectious agents related to gastric carcinogenesis — is higher in patients from southwest Mexico compared to other populations. The EBV/HCMV coinfection was the most frequent in gastritis and gastric cancer patients and an important proportion of them presented *H. pylori*/EBV or *H. pylori*/HCMV coinfection suggesting the possibility of a synergic effect during chronic inflammation and carcinogenesis. The presence of EBV in malignant tumors is independent of the cancer type. It is necessary to explore the host factors and the possible interaction mechanisms between *H. pylori* and EBV or HCMV, as well as those between EBV and HCMV. The question that remains to resolve is if *H. pylori* induces the EBV lytic phase and if it favors the reactivation of latent infection by HCMV.

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