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Colonization of Mexican Patients by Multiple *Helicobacter pylori* Strains with Different *vacA* and *cagA* Genotypes

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***Helicobacter pylori* virulence determinants have not previously been studied in detail in Latin Americans with *H. pylori* infections. We characterized the *vacA* (vacuolating cytotoxin gene A) and *cagA* (cytotoxin-associated gene A) types of more than 400 single-colony isolates from 20 patients in Mexico City. For 17 patients *H. pylori* strains of two or more different *vacA* genotypes were isolated from gastric biopsy specimens, indicating infection with two or more strains of *H. pylori*. The most frequent *vacA* genotype was s1b/m1. *vacA* diversity was more marked than that described previously, in that isolates from seven patients had untypeable *vacA* midregions and isolates from nine patients had type s1 signal sequence coding regions which could not be further subtyped. Previously undescribed *vacA* type s2/m1 strains were found in five patients. All patients were infected with *cagA*-positive strains, but occasionally, these coexisted with small numbers of *cagA*-negative strains. In conclusion, coinfection with multiple *H. pylori* strains is common in Mexico, and *vacA* in these strains is genetically more diverse than has been described in other populations.**

Helicobacter pylori infection is the main cause of duodenal and gastric ulceration (4, 25, 27) and is an important risk factor for the development of distal gastric adenocarcinoma and gastric lymphoma (9, 28). However, most people infected with *H. pylori* do not develop these conditions in their lifetimes. One factor that affects who develops disease is bacterial virulence. Two bacterial virulence determinants linked with disease are the possession of a pathogenicity island, for which the gene *cagA* (cytotoxin-associated gene A) is a marker (5, 6, 12), and production of a cytotoxin which induces vacuolation of cultured epithelial cell in vitro (10, 14, 18, 23, 29). Although all *H. pylori* strains possess the gene encoding the cytotoxin, *vacA*, only about 40% of U.S. strains express HeLa cell vacuolating activity in vitro (7, 32). Analysis of *vacA* from different U.S. strains shows that alleles differ, particularly in their midregions, and these alleles may be one of two types (m1 or m2). The alleles in their signal sequence regions also differ, and there may be one of three types (s1a, s1b, or s2). All combinations of *vacA* signal sequence and midregion allele types have been described except s2/m1 (2). In a U.S. study, the *vacA* genotype was associated with cytotoxin activity in vitro (s1a > s1b > s2 and m1 > m2) and with peptic ulceration (s1a > s1b or s2) (2, 3). Other preliminary studies have supported the finding that *vacA* s2 strains are rarely associated with ulcers but have also found that such strains are uncommon in many populations (17, 20).

In this study, we aimed to assess the *vacA* and *cagA* genotypes of *H. pylori* strains infecting Mexican patients. It quickly became apparent that these patients were infected with multiple strains with different *vacA* genotypes. In this paper we describe in detail the *vacA* and *cagA* genotypes of the multiple strains infecting Mexican patients and the vacuolating activities of pooled groups of strains from each patient. Also, we describe strains which cannot be adequately typed by the published PCR-based system and, for the first time, strains with the *vacA* s2/m1 genotype.

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MATERIALS AND METHODS

Patient characteristics and gastric biopsy specimen collection. We obtained gastric biopsy specimens from 20 unselected *H. pylori*-infected patients (median age, 54 years; age range, 30 to 66 years) undergoing upper gastrointestinal endoscopy at Hospital de Alta Especialidad de Petroleos Mexicanos, Mexico City, Mexico; 6 of these patients had duodenal ulceration, 6 had gastric ulceration, and 8 had nonulcer dyspepsia. From each patient, one biopsy specimen from each of the gastric antrum and the corpus was placed immediately in Stuart's (16) medium and was transported to the laboratory within 3 h. A second biopsy specimen from each site was placed in 10% formalin saline for later histological examination. This was processed routinely, and adjacent sections were stained with hematoxylin and eosin and a modified Giemsa stain. Between endoscopies, endoscopes and biopsy forceps were cleaned thoroughly with detergent, disinfected by submersion in ethanol for 20 min, and then rinsed in sterile water. Ethical approval for the study was obtained from the South Central Hospital Ethical Committee, and all patients gave written informed consent to participate in the study.

***H. pylori* culture and chromosomal DNA extraction.** *H. pylori* was cultured by smearing biopsy specimens on the surfaces of horse blood agar plates (10% horse blood in Casman agar base [BBL Microbiology Systems, Cockeysville, Md.]), which were incubated in 5% oxygen–10% carbon dioxide for 72 h at 37°C for up to 5 days (16, 30). The antral and corpus biopsy specimens were studied separately. Typical colonies were identified as *H. pylori* by morphology following Gram staining (gram-negative spiral or curved rods) and biochemical testing (positive urease, oxidase, and catalase tests). The rest of the colonies were harvested into three batches with a sterile cotton swab. Batch 1 (25% of total colonies) was used for DNA extraction as described previously (1), and this is referred to as the "multiple-colony sample." Batches 2 (25% of colonies) and 3 (50% of colonies) were stored at −70°C in 1.5 ml of brucella broth (BBL Microbiology Systems) with 10% fetal calf serum and 15% glycerol until use. Batch 2 was used for later assessment of vacuolating cytotoxin activity, as described below. Batch 3 was later thawed and recultured on blood agar plates as described above. Between 9 and 12 colonies were picked separately from these plates and were then passed onto individual plates. Chromosomal DNA was extracted from these "single-colony isolates" as described above. Great care was taken at all times not to cross-contaminate samples.

***vacA* and *cagA* genotyping by specific PCR amplification.** The *vacA* signal sequence and midregion were typed by allelic type-specific PCR as described previously (2). In brief, each strain was typed as *vacA* signal region type s1a, s1b,

or s2 by performing three separate PCR assays, each with a different allelic type-specific forward primer (based on the difference in the region encoding the second half of the *vacA* signal sequence) and a common reverse primer. As a check, each strain was also typed with conserved forward and reverse primers designated to amplify a product from all *vacA* alleles, but a larger product was obtained from s2 alleles than from s1a and s1b alleles (the last two alleles cannot be differentiated by this method). Product sizes were differentiated on a 2% agarose gel. The *vacA* midregions were typed as m1 or m2 by performing two separate PCR assays, each with two allelic type-specific primers. Conditions for thermal cycling were 35 cycles of 94°C for 60 s, 52°C for 60 s, and 72°C for 60 s. PCR detection of *cagA* was as described above, except that annealing was at 59°C and a 10-min elongation step was used after the final cycle (22).

Quantitative assessment of vacuolating activity. Stored samples (from batch 2, described above) infected with strains of different *vacA* types (multiple-colony samples) were recultured as described earlier. A swab of multiple colonies was then used to inoculate 25 ml of brucella broth with 10% fetal calf serum. Culture was for 72 h under microaerobic conditions (as described above) with gentle agitation. Culture supernatants were concentrated 40-fold by ultrafiltration as described previously (8, 11). These concentrated culture supernatants were incubated with HeLa cells in twofold serial dilutions from a total of 1:5 to 1:160 as described previously (11, 13, 24). Cells were observed for up to 48 h for the development of vacuoles. The maximum dilution of the sample that produced vacuolation in more than 30% of the HeLa cells was defined as the cytotoxin activity score for a sample. Uninoculated broth was used as a negative control, and broth culture concentrates from strains 60190 (7) and Tx30a (2, 7) were used as positive vacuolating and negative vacuolating controls, respectively.

RESULTS

Histology. *H. pylori* isolates were seen in all antral and all corpus biopsy specimens. All biopsy specimens showed polymorphonuclear and round cell infiltration.

***vacA* and *cagA* typing of multiple-colony samples.** More than one *vacA* allelic type was identified in 18 of the DNA specimens from the 40 multiple-colony samples and in a total of 13 of 20 patients. For the *vacA* signal region, type s1b alleles were most common and were found in 31 of 40 samples and 16 of 20 patients. Type s2 alleles were found in 12 of 20 samples and 9 of 20 patients, and type s1a alleles were found in both antral and corpus biopsy specimens from 2 of 20 patients (Table 1). In total, 10 of 40 multiple colony samples contained more than one *vacA* signal sequence type and 8 of 20 patients were infected with at least two strains with different *vacA* signal region types. Regarding the *vacA* midregion, type m1 alleles were found in both antral and corpus biopsy specimens from 19 of 20 patients, and type m2 alleles were found in 15 of 40 samples and in 10 of 20 patients. Type m2 alleles were always found with m1 alleles except in one patient whose antrum and corpus were both infected with pure m2 strains. No relationship between *vacA* allelic type and preference for antral or corpus site of gastric colonization was found. No relationship between the sequence of endoscopy and the occurrence of multiple *H. pylori vacA* types was found.

Vacuolating cytotoxin activity of multiple-colony samples. Of the 40 multiple-colony samples assessed for vacuolating cytotoxin activity, broth culture supernatants from 33 caused vacuolation in HeLa cells (Table 1). The seven samples that did not produce vacuolating activity were from five patients infected with strains of different *vacA* types, but all strains had type s2 and/or type m2 *vacA* alleles. Of the 33 cytotoxic samples, supernatants from 4 produced vacuolation in more than 80% of HeLa cells at all dilutions. All four of these samples contained *vacA* s1a or s1b and m1 isolates (Table 1).

***vacA* and *cagA* typing of single-colony isolates.** To try to explain the finding of multiple *vacA* genotypes in single biopsy specimens, we took between 9 and 12 single colonies from both the antrums and the corpora of all 20 patients and typed these single-colony isolates. In 12 of 20 corpus specimens and 14 of 20 antral specimens, we found strains with different *vacA* types, and data for 26 of 40 of these specimens are shown in Table 2. As expected, the single-colony isolates and single *vacA* signal

TABLE 1. *vacA* and *cagA* genotyping and cytotoxic activity based on multiple-colony sweeps from primary culture plates[a]

Subject no.	Endoscopic diagnosis	Site	*cagA* status	*vacA* allelic type detected		Vacuolating activity titer[b]
				Signal region	Midregion	
1	No ulcer	a	+	s1b	m1, m2	1.3
		c	+	s1b	m1, m2	1.3
2	No ulcer	a	+	s1b	m1, m2	1.3
		c	+	s1b, s2	m1	1.3
3	GU	a	+	s1b	m1, m2	1.6
		c	+	s1b, s2	m1, m2	1.3
4	DU	a	+	s1b, s2	m1, m2	1.3
		c	+	s1b, s2	m1, m2	1.3
5	DU	a	+	s1b, s2	m1	1.6
		c	+	s1b, s2	m1	2.2[c]
6	DU	a	+	s1b, s2	m1	2.2[c]
		c	+	s1b	m1	0.7
7	No ulcer	a	+	s1b	m1	2.2[c]
		c	+	s1b	m1	1.6
8	No ulcer	a	+	s1b	m1	1.3
		c	+	s1b	m1, m2	—[d]
9	DU	a	+	s1b, s2	m1, m2	—
		c	+	s1b	m1	1.3
10	DU	a	+	s1b	m1	0.7
		c	+	s1b	m1	0.7
11	GU	a	+	s1b	m1	1.6
		c	+	s1b	m1	1.6
12	GU	a	+	s1b, s2	m1	—
		c	+	s1b	m1	1.6
13	GU	a	+	s1b	m1, m2	—
		c	+	s1b, s2	m1, m2	—
14	GU	a	+	s1b	m1	1.6
		c	+	s1b	m1	1.6
15	No ulcer	a	+	s1[e]	m1	1.3
		c	+	s1	m1	1.3
16	No ulcer	a	+	s1b	m1	1.6
		c	+	s1	m1	1.3
17	No ulcer	a	+	s1b	m1	1.9
		c	+	s1b	m1, m2	1.6
18	DU	a	+	s1a	m1	1.3
		c	+	s1a	m1, m2	1.3
19	GU	a	+	s1a	m1	2.2[c]
		c	+	s1a	m1	1.6
20	No ulcer	a	+	s2	m2	—
		c	+	s2	m2	—

[a] Abbreviations: DU, duodenal ulcer; GU, gastric ulcer; a, antrum; c, corpus.
[b] Expressed as $\log_{10}$ dilutions of 40-fold-concentrated broth culture supernatants which induced vacuolation in >30% semiconfluent HeLa cells.
[c] These samples induced vacuolation in >80% of HeLa cells at all dilutions.
[d] —, these samples did not cause HeLa cell vacuolation.
[e] When the *vacA* allelic type is designated s1, the s1a and s1b subtypes could not be determined.

TABLE 2. *cagA* and *vacA* genotypes of single-colony isolates from patients with multiple infection[a]

Subject no.	Site[b]	No. of colonies tested	No. of *cagA*-positive colonies	*vacA* allelic types detected (no. of isolates)
1	a	10	8[c]	s1b/m1 (8), s1b/m2 (2)
	c	10	10	s1b/m1 (9), s1b/m2 (1)
2	a	9	9	s1b/m1 (5), s1b/m2 (4)
	c	10	9[c]	s1b/m1 (6), s2/m1 (1), s1[d]/m1 (2), s1b/m0[e] (1)
3	a	10	10	s1b/m1 (6), s1b/m2 (2), s1/m1 (2)
	c	10	10	s1b/m1 (7), s2/m1 (2), s1b/m2 (1)
4	a	11	11	s1b/m1 (10), s2/m2 (1)
	c	10	10	s1b/m1 (8), s2/m2 (2)
5	a	10	10	s1b/m1 (9), s2/m1 (1)
	c	11	10[f]	s1b/m1 (8), s2/m1 (2), s1b/m0 (1)
6	a	11	11	s1b/m1 (7), s2/m1 (3), s1/m1 (1)
8	c	10	10	s1b/m1 (7), s1b/m2 (3)
9	a	10	10	s1b/m1 (6), s1b/m2 (1), s2/m2 (3)
12	a	10	10	s1b/m1 (9), s2/m1 (1)
13	a	10	10	s1b/m1 (8), s1b/m2 (2)
	c	10	10	s1b/m1 (6), s1b/m2 (2), s2/m2 (2)
14	c	10	10	s1b/m1 (8), s1/m1 (2)
15	a	10	10	s1/m1 (9), s1/m0 (1)
16	a	10	10	s1b/m1 (6), s1/m1 (4)
	c	10	8[g]	s1b/m1 (8), s1/m1 (2)
17	a	10	10	s1b/m1 (8), s1/m1 (1), s1b/m0 (1)
	c	10	10	s1b/m1 (8), s1/m1 (1), s1b/m2 (1)
18	c	10	8[g]	s1a/m1 (3), s1a/m2 (4), s1/m1 (3)
19	a	12	12	s1a/m1 (7), s1a/m0 (2), s1/m1 (3)
	c	10	10	s1a/m1 (7), s1/m1 (3)
20	a	10	7[h]	s2/m2 (7), s1/m2 (2), s2/m0 (1)

[a] Data for patients or biopsy specimens not infected with strains of multiple type are not included.
[b] a, antrum; c, corpus.
[c] The *cagA*-negative colonies from these biopsy specimens were *vacA* s1b/m1.
[d] When the *vacA* allelic type is designated s1, the s1a and s1b subtypes could not be determined.
[e] When the *vacA* allelic type is designated m0, the midregion type could not be determined.
[f] The *cagA*-negative colony from this biopsy specimen was *vacA* s2/m1.
[g] The *cagA*-negative colonies were *vacA* s1/m1.
[h] One *cagA*-negative colony was *vacA* s2/m0 and two were *vacA* s2/m2.

and midregion types, and we assume that they represent single strains. For three patients (patients 7, 10, and 11) colonies of only a single *vacA* signal and midregion type were found in both the antrums and corpora, for three patients colonies of a single type were found in the antrums and mixed colonies were found in the corpora, for five patients mixed colonies were found in the antrums and single *vacA* types were found in the corpora, and for nine patients mixed colonies were found in both the antrums and the corpora. Five patients were infected with strains with the *vacA* s2/m1 genotype, which has not previously been found to occur naturally. By typing single-colony isolates, we found multiple *vacA* types in five patients in whom this had not been expected from the typing of the multiple-colony samples. Strains which were *vacA* s1 were found in nine patients, but these could not be subtyped as *vacA* s1a or s1b. Strains for which the *vacA* midregion could not be typed as m1 or m2 were found in seven patients. *cagA*-positive strains predominated and were found in both the antrums and corpora of all 20 patients. However, six patients also had low numbers of *cagA*-negative isolates: for four patients in the corpus biopsy specimen and for two patients in the antral biopsy specimen. Overall, infection with two or more strains with different *vacA* genotypes was found in 17 of 20 patients.

DISCUSSION

This paper provides the first detailed description from a developing country of *Helicobacter pylori* typing with the virulence marker genes *vacA* and *cagA*. The first important finding is that single biopsy specimens from Mexican patients are infected with strains of multiple *vacA* types. That our findings were not due to a poor specificity of PCR amplification is confirmed by the finding that single-colony isolates gave single *vacA* signal region and midregion types on every occasion. When biopsy specimens from different gastric sites in the same patient were also considered, all but three of our patients had evidence of infection with two or more strains with different *vacA* genotypes. The methodology that we used will underestimate infections with multiple types of strains, as two biopsy specimens do not adequately sample the whole stomach. Multiple-strain infection is well described in developed countries (15, 19, 21, 26, 31), but it is not clear why it is so much more extensive in Mexican patients. The coexistence of strains with different *vacA* genotypes could be explained in several different ways. First, different *vacA* and *cagA* genotypes might offer no competitive advantage to strains. Second, the different genotypes could confer different advantages that allow the strains to survive in slightly different ecological niches within a gastric biopsy specimen. Finally, multiple continuing infections with strains of different genotypes may be occurring. The final possibility would have important implications for reacquisition of infections after treatment.

In the Mexican context of multiple infections with strains of different *vacA* genotypes, we have shown that there is more diversity in the *vacA* signal and midregions than was described previously (2), in that some strains had *vacA* alleles which were not fully typeable with previously described PCR primers. We also describe, for the first time, alleles with the s2/m1 genotype. This is an important finding, as not being able to find such strains previously had been an argument for clonal expansion of *H. pylori* populations and against the occurrence of recombination between *H. pylori* strains within *vacA* in vivo. The finding of all combinations of the signal sequence and the midregion in *vacA* is much more consistent with the results of multilocus enzyme electrophoresis studies (15), which suggested frequent past recombination between *H. pylori* strains. Recombination within *vacA* may also explain why *vacA* alleles from other strains are not readily typeable, although genetic diversity generated by mutation could explain this equally well.

Analysis of vacuolating cytotoxin activities of the primary cultures of strains of mixed *vacA* types in this study is consistent with the previous description of the different vacuolating activities of strains of different *vacA* genotypes (2, 3). *vacA* s1b/m1 strains are described as having lower vacuolating activity than *vacA* s1a/m1 strains, and the vacuolation in 30 to 80% of HeLa cells described here for most specimens infected with strains of multiple *vacA* types is less than the vacuolation in

>80% described for s1a/m1 strains from the United States (2, 3). Isolates from five patients in this study did not cause vacuolating activity, and all had *vacA* s2 and/or m2 alleles, which have previously been shown to confer little or no vacuolating activity (2). The samples from four patients infected with strains of mixed *vacA* types induced vacuolation in >80% of HeLa cells at all dilutions, and these samples contained s1a or s1b/m1 *vacA* strains, which have previously been shown to be associated with higher levels of cytotoxin activity (2). Heterogeneity between samples in terms of vacuolating activity was evident, but this is to be expected, as there is marked heterogeneity between strains with identical *vacA* genotypes (2), and additionally, the relative contributions of individual strains in these specimens infected with strains of multiple *vacA* types will vary.

This study has important implications for the pathogenesis and clinical management of *H. pylori* infection in Mexico and other developing countries. Studies from the United States that link infection with strains of different *vacA* and *cagA* genotypes with gastroduodenal disease are not directly applicable to populations like that in Mexico, in which infection with multiple strains of different *vacA* and *cagA* genotypes is common. Whether these "virulence markers" have any impact on virulence in this setting remains to be determined. How infections with strains of different genotypes have arisen, in particular, whether they are acquired in childhood or throughout adult life, is also of considerable clinical importance and requires urgent further study. The important implication for treatment would be that antibiotic treatment of *H. pylori* infection would not be an effective clinical strategy in individual patients if they were immediately reinfected with another strain. Furthermore, if vaccines became available, continuing infection in adult life would mean that these would have to provide lifelong protection to be effective and would need to be polyvalent for effective protection.

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