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Comparison of Genotyping *Helicobacter pylori* Directly from Biopsy Specimens and Genotyping from Bacterial Cultures

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PCR for *vacA* and *cagA* genotypes of *Helicobacter pylori* using DNA isolated from infected gastric biopsy specimens was approximately equal to genotyping using bacterial DNA from cultures. Inconsistent results were associated with low *H. pylori* density in biopsies. A higher proportion of mixed infection was found when biopsies were used.

Peptic ulcer or gastric cancer is most likely associated with *cag* pathogenicity island (PAI)-positive *Helicobacter pylori* strains (3, 4). Although attempts to associate specific mosaic combinations of signal sequences (s1a, s1b, s1c, and s2) and middle region allelic types (m1 and m2) of the *vacA* gene have met with little success, there is interest in genotyping *H. pylori* in relation to this factor (1, 8, 11). This study was undertaken to determine whether genotyping with DNA extracted from gastric biopsy specimens provided results similar to those with bacterial DNA isolated from *H. pylori* cultured from gastric biopsies.

DNA from serial dilutions of *H. pylori* cells (0 to 10^6 bacteria per PCR) from strains ATCC 43504 (*vacA* s1a-m1, *cagA* positive), ATCC 51932 (*vacA* s2-m2, *cagA* negative) and C90 (*vacA* s1b-m1, *cagA* positive) was used to define the accuracy of the *cagA* and *vacA* PCR assays (Table 1). PCR amplification was performed as previously described (11). PCR products with the expected band size were regarded as positive for the target genes. PCR for the *cag* empty site was used to confirm the absence of the entire *cag* PAI (6). If both *cagA* and empty site PCRs were positive, the sample was scored as a mixed *cagA*-positive and *cagA* (or *cag* PAI)-negative infection. If both *cagA* and empty site PCRs were negative, the samples were recorded as having no band (NB). Genotyping with bacterial DNA was possible with as few as 10 bacteria per PCR, although the *cag* empty site required 100 bacteria per PCR (Table 2). The specificity of the PCR assay was confirmed with 37 related and unrelated bacteria, including 3 *Helicobacter* species and 4 *Campylobacter* species (5). All primers proved specific for *H. pylori.*

We simulated mucosal biopsies by adding approximately 50 mg of noninfected gastric mucosal tissues to each of 96 wells of a microtiter plate along with serial dilutions of *H. pylori* suspended in RPMI 1640 medium (Invitrogen Corp., Carlsbad, Calif.). After 2 h of incubation at 37°C with 5% CO_2 to enhance attachment and bacterial tissue interactions, samples in the plates were centrifuged at 12,000 × *g* for 1 min, the supernatants were discarded, and DNA was extracted from the pellets by using the QIAamp tissue kit (QIAGEN, Inc.) (spiked-tissue experiment). Tissue DNA without added *H. pylori* served as negative controls, and no PCR bands were detected, again confirming specificity for *H. pylori.* With the exception for genotypes of *vacA* s, *vacA* s1a, and the *cag* empty site, PCR using simulated biopsy tissue required a minimum of 10 times more DNA than genotyping using bacterial DNA (Table 2).

We simulated mixed infection by adding approximately 50 mg of noninfected gastric mucosal tissues to each of 96 wells along with two different genotypes of *H. pylori* (10^3 to 10^5 CFU of each strain) suspended in RPMI 1640 medium (Invitrogen). This number of bacteria was based on our prior experience and published data showing that the concentration of *H. pylori* in gastric mucosal biopsies was typically in the range of 5×10^3 to 5×10^5 bacteria per biopsy sample (2, 10). We used two *vacA* s1a-m1, *cagA*-positive strains (ATCC 43504 and JK31), two *vacA* s1b-m1, *cagA*-positive strains (C90 and C91), and two *vacA* s2-m2, *cagA*-negative strains (ATCC 51932 and C94) for the mixed spiked-tissue experiments. For each genotype, we tested one fast-growing strain and one slow-growing strain based on the time required to reach a plateau in liquid culture.

Using a starting bacterial dose of approximately 10^6 bacteria, fast growers required approximately 10 h, and slow growers required approximately 30 h to reach the same cell density. Samples were handled as described above (in vitro spiked-tissue experiment). Homogenized tissues were cultured before DNA extraction, and DNA corresponding to 10^6 bacteria from multiple colonies was used for each PCR. In the mixed spiked-tissue experiment, we could detect both genotypes irrespective of the rate of growth and the starting dilution (10^3 and 10^5 [1:100] to 10^5 and 10^3 [100:1]). However, genotyping results following coculture of fast- and slow-growing strains was observed to be dependent on both the rate of growth and the starting dilution (Table 3). Only when the before-culture growth ratio (slow/fast) of the two strains was 1% or less were the results other than "mixed culture." When we used two

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TABLE 1. PCR primers for amplification of *cagA* and *vacA* sequences

Gene and DNA region amplified	Primer	Primer sequence (5′→3′)[a]	PCR product size (bp)	Reference
cagA	CAGAF	GATAACAGGCAAGCTTTTGAGG	349	11
	CAGAR	CTGCAAAAGATTGTTTGGCAGA		11
cagA	CAGA5F	GGCAATGGTGGTCCTGGAGCTAGGC	325	6
	CAGA5R	GGAAATCTTTAATCTCAGTTCGG		6
cag empty site	Luni1	ACATTTTGGCTAAATAAACGCTG	550	6
	R5280	GGTTGCACGCATTTTCCCTTAATC		6
vacA s1	VA1-F	ATGGAAATACAACAAACACAC	259	1
	VA1-R	CTGCTTGAATGCGCCAAAC		1
vacA s2	VA1-F	ATGGAAATACAACAAACACAC	286	1
	VA1-R	CTGCTTGAATGCGCCAAAC		1
vacA s1a	S1A-F[b]	TCTYGCTTTAGTAGGAGC	212	9
vacA s1b	SS3-F[b]	AGCGCCATACCGCAAGAG	187	1
vacA m1	VAG-F	CAATCTGTCCAATCAAGCGAG	570	11
	VAG-R	GCGTCTAAATAATTCCAAGG		11
vacA m2	VAG-F	CAATCTGTCCAATCAAGCGAG	645	11
	VAG-R	GCGTCTAAATAATTCCAAGG		11

[a] Y = C + T.
[b] Used with primer VA1-R.

strains with the same growth speed (fast or slow), we could correctly genotype the strains irrespective of the starting ratios, which varied between 1:100 and 100:1.

We finally examined the gastric mucosal biopsy specimens from 166 *H. pylori* culture-positive Colombian patients in which mixed infection is common (9, 12). Informed consent was obtained from all patients, and the ethics committee of Universidad Nacional de Colombia approved the protocol. Antral biopsy specimens were sent frozen on dry ice to Houston, where they were defrosted and homogenized by grinding. The wet weights were approximately 50 mg in each sample. Genomic DNA was directly extracted from biopsy specimens as described for the spiked-tissue experiments. The homogenized material was also cultured, and DNA from multiple colonies was extracted as described above. Again, DNA corresponding to 10^6 bacteria per PCR was used. There was good agreement between PCR-based genotyping results with tissue DNA and those with bacterial DNA, especially for *vacA* m1 versus m2 and *vacA* s1 versus s2 (Table 4). Inconsistencies between results from PCR from the biopsy samples and culture of the sample were possibly due to differences in growth rate among the strains present in the stomach or the numbers of organisms per strain. The interpretation of the results to determine which approach was superior depended on how one handled the data regarding mixed infections. Use of PCR from the biopsy specimen showed 32 cases with mixed *vacA* s1a and s1b strain, whereas PCR from bacteria recovered by culture revealed only 10 cases. Overall, we detected mixed infection

TABLE 2. Limits of detection of each primer in two different specimens for PCR

DNA	Limit of detection (CFU/PCR) for[a]:						
	vacA		*cagA*		*cag* empty site	*vacA*	
	s1/s2	m1/m2	5′ region	Middle region		s1a	s1b
Bacterial	10	10	10	10	100	10	10
Bacteria/tissue	10	100	100	100	100	10	100

[a] Each value represents the minimum amount of *H. pylori* (CFU) per PCR that could be detected by PCR.

TABLE 3. Effects of mixed infection in in vitro study of different concentrations of each strain

Mixed culture[a]	Result for strain concn at ratio[b]:				
	1:1 (10^4:10^4)	1:10 (10^3:10^4)	1:100 (10^3:10^5)	10:1 (10^4:10^3)	100:1 (10^5:10^3)
vacA s1-m1-*cagA*+ (slow)–*vacA* s2-m2-*cagA*− (fast)					
s	Mixed	Mixed	s2	Mixed	Mixed
m	Mixed	Mixed	s2	Mixed	Mixed
cagA	Mixed	Mixed	Negative	Mixed	Mixed
vacA s2-m2-*cagA*− (slow)–*vacA* s1-m1-*cagA*+ (fast)					
s	Mixed	Mixed	s1	Mixed	Mixed
m	Mixed	Mixed	s1	Mixed	Mixed
cagA	Mixed	Mixed	Positive	Mixed	mixed
vacA s1b-m1-*cagA*+ (slow)–*vacA s1a-m1-cagA*+ (fast)					
s1 subtype	Mixed	Mixed	s1a	Mixed	Mixed
vacA s1a-m1-*cagA*+ (slow)–*vacA* s1b-m1-*cagA*+ (fast)					
s1 subtype	Mixed	Mixed	s1b	Mixed	Mixed

[a] +, positive; −, negative.
[b] Ratio of CFU (in parentheses) from each strain when we initially made a mixture of two strains.

TABLE 4. PCR results from tissue DNA compared with culture DNA

Genotype (*n*)	No. (%) with NB by biopsy	No. of mixed infections by biopsy	No. (%) with identical result for mixed infections	No. of inconsistent cases (different genotypes)	No. (%) with identical result	Overall accuracy (%)[a]
cagA (166)	14 (8.4)	3	0 (0)[b]	1	148 (97.4)[c]	89.2
vacA m1, 2 (166)	5 (3.0)	9	5 (55)	1	156 (96.9)	94.0
vacA s1, 2 (166)	1 (0.6)	4	0 (0)	1	160 (97.0)	96.4
vacA s1a, b (142)[d]	4 (2.8)	32	10 (31)	1	115 (83.3)	81.0

[a] Correct determination of status (including those with NB found as false-negative results).
[b] Percentage in which mixed infection results were identical between the two methods.
[c] Percentage in which results were identical between the two methods (excluding those with NB).
[d] Does not include s2 genotypes.

(defined as evidence of two strains with any of the genes examined) in 27% of cases when we used tissue DNA compared to only 9% when we used bacterial DNA. In gastric cancer specimens, there was only fair-to-moderate agreement in genotyping between tissue DNA and bacterial DNA for the *vacA* s1 subtype, because there were many cases with mixed infection of s1a and s1b genotypes in tissue DNA (19 of 51 [37%] versus 3 of 51 [6%], respectively). Thus, studies to identify relationships between virulence factors and disease presentation (e.g., gastric cancer) should be restricted to sites where mixed infection is uncommon. However, excluding mixed infections when they are common might produce a misleading perception of the actual relationships of bacterial strains to pathology of the diseases (8).

Direct PCR from biopsy specimens tended to underestimate the prevalence of a specific virulence factor such as *cagA* (e.g., in 14 cases, or 8.4%, NB was detected with *cagA*, although the culture showed infection with a *cagA*-positive strain) (Table 4). All of these cases had low *H. pylori* density by histology (data not shown), suggesting that low *H. pylori* density in biopsy specimens may result in misleading results with direct PCR. Partial deletion of the *cag* PAI could also yield *cagA*-negative and *cag* empty-site-negative results (i.e., NB). However, because bacterial DNA yielded positive *cagA* results for the same cases, the possibility of a partially deleted *cag* PAI is thought to be rare.

Cases with inconsistent genotypes were fortunately rare (Table 4): all such cases had very low *H. pylori* density by histology, and the positive bands were faint (data not shown), suggesting that there was insufficient DNA from the biopsy samples to correctly genotype these cases. Direct PCR analysis of gastric biopsy specimens is less tedious and time-consuming than culturing of the strains, and it is theoretically possible to perform genotyping from formalin-fixed paraffin-embedded gastric biopsy specimens (7). Clinically, it would be important to include positive and negative controls to exclude the possibility of inhibitors of the PCR in the tissue sample. The use of real-time PCR or Southern hybridization might also provide improved results.

REFERENCES

1. **Atherton, J. C., P. Cao, R. M. Peek, M. K. R. Tummuru, M. J. Blaser, and T. L. Cover.** 1995. Mosaicism in vacuolating cytotoxin alleles of *Helicobacter pylori*. J. Biol. Chem. **270:**17771–17777.
2. **Atherton, J. C., K. T. Tham, R. M. Peek, T. L. Cover, and M. J. Blaser.** 1996. Density of *Helicobacter pylori* infection in vivo as assessed by quantitative culture and histology. J. Infect. Dis. **174:**552–556.
3. **Blaser, M. J., G. I. Perez-Perez, H. Kleanthous, T. I. Cover, R. M. Peek, and P. H. Chyou.** 1995. Infection with *Helicobacter pylori* strains possessing *cagA* is associated with an increased risk of developing adenocarcinoma of the stomach. Cancer Res. **55:**2111–2115.
4. **Censini, S., C. Lange, Z. Xiang, J. E. Crabtree, P. Ghiara, M. Borodovsky, R. Rappuoli, and A. Covacci.** 1996. *cag*, a pathogenicity island of *Helicobacter pylori*, encodes type I-specific and disease-associated virulence factors. Proc. Natl. Acad. Sci. USA **93:**14648–14653.
5. **Hulten, K., S. W. Han, H. Enroth, P. D. Klein, A. R. Opekun, R. H. Gilman, D. G. Evans, L. Engstrand, D. Y. Graham, and F. A. El-Zaatari.** 1996. *Helicobacter pylori* in the drinking water in Peru. Gastroenterology **110:**1031–1035.
6. **Mukhopadhyay, A. K., D. Kersulyte, J.-Y. Jeong, S. Datta, Y. Ito, A. Chowdhury, S. Chowdhury, A. Sandra, S. K. Bhattacharya, T. Azuma, B. Nair, and D. E. Berg.** 2000. Distinctiveness of genotypes of *Helicobacter pylori* in Calcutta, India. J. Bacteriol. **182:**3219–3227.
7. **Scholte, G. H. A., L. J. van Doorn, W. G. V. Quint, and J. Lindeman.** 2001. Genotyping of *Helicobacter pylori* strains in formalin-fixed or formaledhyde-sublimate-fixed paraffin-embedded gastric biopsy specimens. Diagn. Mol. Pathol. **10:**166–170.
8. **van Doorn, L. J., C. Figueriedo, R. Sanna, A. Plaisier, P. Schneeberger, W. D. Boer, and W. Quint.** 1998. Clinical relevance of the *cagA*, *vacA*, and *iceA* status of *Helicobacter pylori*. Gastroenterology **115:**58–66.
9. **Yamaoka, Y., T. Kodama, O. Gutierrez, J. G. Kim, K. Kashima, and D. Y. Graham.** 1999. Relationship between *Helicobacter pylori iceA*, *cagA*, and *vacA* status and clinical outcome: studies in four different countries. J. Clin. Microbiol. **37:**2274–2279.
10. **Yamaoka, Y., T. Kodama, M. Kita, J. Imanishi, K. Kashima, and D. Y. Graham.** 1999. Relation between clinical presentation, *Helicobacter pylori* density, interleukin-1β and -8 production, and *cagA* status. Gut **45:**804–811.
11. **Yamaoka, Y., T. Kodama, M. Kita, J. Imanishi, K. Kashima, and D. Y. Graham.** 1998. Relationship of *vacA* genotypes of *Helicobacter pylori* to *cagA* status, cytotoxin production, and clinical outcome. Helicobacter **4:**241–253.
12. **Yamaoka, Y., H. M. T. El-Zimaity, O. Gutierrez, N. Figura, J. G. Kim, T. Kodama, K. Kashima, and D. Y. Graham.** 1999. Relationship between subtypes of the *cagA* 3′ repeat region, gastric histology, and susceptibility to low pH. Gastroenterology **117:**342–349.