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Molecular Basis of Pathogenicity in *Helicobacter pylori* Clinical Isolates[▽]

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This study identified pathogenicity genes in 40 *Helicobacter pylori* clinical isolates. The *cagA*, *vacA*, and *iceA* genes were detected in 65%, 97.5%, and 97.5% of the isolates, respectively. The *cagA*, *iceA1*, and *vacAs1a*/*m1* genes were related to erosive gastritis, whereas the *vacAs2*/*m2* and *iceA2* genes were associated with enanthematous gastritis.

Helicobacter pylori is considered the major etiologic agent of chronic active gastritis, an essential catalyst in the emergence of peptic ulcer, and a risk factor for the development of gastric cancer (17). Studies indicate that the evolution of the infection depends in part on the expression of specific bacterial pathogenicity genes, such as *cagA* (*c*ytotoxin-*a*ssociated *g*ene A), *vacA* (*vac*uolating cytotoxin), and *iceA* (*i*nduced by *c*ontact with *e*pithelium) (2).

The *cagA* gene is considered to be a marker for the presence of a *cagA* pathogenicity island (8). The *cagA*-positive *H. pylori* strains increase interleukin-8 production and gastric inflammation (5). The *vacA* gene encodes a vacuolating cytotoxin able to induce the formation of cytoplasmic vacuoles in epithelial cells (11). This gene comprises two variable regions: the signal region, with two alleles, *s1* (subtypes *s1a*, *s1b*, and *s1c*) and *s2*, and the middle region, with the alleles *m1* and *m2* (3, 28). In general, the *s1*/*m1* strains produce large amounts of vacuolating cytotoxin, the *s1*/*m2* strains produce moderate amounts, and the *s2*/*m2* strains produce little or none (3). The *iceA* gene has two alleles: *iceA1* and *iceA2*. The *iceA1* allele is associated with peptic ulcer, and *iceA2* is related to asymptomatic gastritis (24, 29).

This study analyzed the presence of *cagA*, *vacA*, and *iceA* genes in clinical isolates and correlated these findings with the endoscopic diagnosis. Forty isolates of *H. pylori* were obtained from biopsy specimens of the gastric antrum collected from dyspeptic patients admitted to the upper gastrointestinal endoscopic ward in the Hospital of the Federal University of Rio Grande, Rio Grande do Sul, Brazil. This study was approved by the ethics committee of our university. Informed consent was obtained from all patients.

After collection, the biopsy specimens were kept in brain heart infusion broth (Acumedia, United States) with 20% glycerol and refrigerated (4 to 8°C) for a maximum of 4 h (22). This broth was thereafter vortexed, and 200 μl was added to medium Columbia agar (Oxoid, United Kingdom), supplemented with 7% sheep blood and with a selective mixture for *Helicobacter* species isolation (Cefar, Brazil). The agar plates were incubated under microaerophilic conditions (5 to 15% O_2 and 10% CO_2) at 37°C for 4 to 10 days (14). The identification of *H. pylori* was performed using catalase, oxidase, and urease tests, microscopy, and *ure*A gene detection (12, 19).

The DNA extraction was performed after 48 h of bacterial growth. Colonies were collected and resuspended in 500 μl of 1× TE buffer. The suspension was centrifuged at 10,000 × *g* for 5 min, and the supernatant was thereafter discarded. The DNA from the clinical isolates was then extracted with DNAzol reagent (Invitrogen, United States) by the method of the manufacturer.

The presence of the *ureA*, *cagA*, *vacA*, and *iceA* genes in the isolates was investigated by PCR using the primers described previously (6, 10, 21, 31). The PCR was performed as described by Rota et al. (for the *ureA* and *cagA* genes) and by Benenson et al. (for the alleles of the *vacA* and *iceA* genes) (4, 27).

The statistical analysis was performed by using Fisher's exact test, a chi-squared test, and a chi-squared test for linear trend. *P* values of less than 0.05 were considered statistically significant.

The presence of the pathogenicity genes was studied in 40 clinical isolates of *H. pylori*. From those, 50% (20 of 40) were obtained from patients with endoscopic diagnosis of enanthematous gastritis and 50% (20 of 40) were obtained from patients with erosive gastritis.

The *cagA* gene was identified in 65% (26 of 40) of the isolates. This frequency is similar to that found in previous studies of *cagA* in Brazil (14, 16, 18). The *vacA* and *iceA* genes were detected in 97.5% (39 of 40) of the samples. The *vacAs1b* (43.6%) and *vacAm2* (53.9%) alleles were the most frequently detected in the 39 isolates, as well as the *iceA2* allele (71.8%). This is an expected result, because these alleles have been reported in other studies (7, 18, 26). Moreover, 12.8% of the isolates verified the presence of the *m1* and *m2* alleles of the *vacA* gene, and 5.1% of the isolates had both *iceA* alleles. The detection of more than one allele of the middle region of *vacA*, as well as the identification of both *iceA* alleles in the same isolate, suggests coinfection of two different strains of *H.*

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TABLE 1. Association between the *cagA* gene and the allelic combinations of the *vacA* gene in isolates of *H. pylori*

Genotype[a]	% with or without *cagA* gene (no. with gene status/total no. of samples)	
	cagA positive	*cagA* negative
vacAs1a/m1	100.0 (5/5)	
vacAs1b/m1	87.5 (7/8)	12.5 (1/8)
vacAs1a/m2	100.0 (5/5)	
vacAs1b/m2	66.7 (4/6)	33.3 (2/6)
vacAs2/m2	10.0 (1/10)	90.0 (9/10)
vacAs1b/m1m2	100.0 (3/3)	
vacAs2/m1m2	50.0 (1/2)	50.0 (1/2)
vacA negative		100.0 (1/1)

[a] $P < 0.001$.

pylori. Cases of patients being infected with multiple strains of *H. pylori* are not uncommon, being more frequent in areas of high *H. pylori* prevalence (9, 15, 23).

The association between the *cagA* and *vacA* genes is described in Table 1. All *cagA*-positive isolates confirmed the presence of *vacA*. The combinations *vacAs1a/m1*, *vacAs1b/m1*, *vacAs1a/m2*, *vacAs1b/m2*, and *vacAs1b/m1m2* were present mainly in *cagA*-positive samples. A statistically significant association was observed between *cagA* and *vacA* ($P < 0.001$).

The relationship of pathogenicity genes with gastric disorders is described in Table 2. The *cagA* gene and the combination *vacAs1a/m1* were frequently detected in isolates from patients with erosive gastritis. Similar findings were reported by other authors (14, 20). These genes are directly related to the infiltration of polymorphonuclear cells, which causes severe epithelial damage. Already, the combination *vacAs2/m2* was frequently observed in isolates from patients with enanthematous gastritis, a finding that suggests that such alleles are related to minor damage in gastric mucosa (1). However, a statistically significant difference was not found in the association between either *cagA* or *vacA* and the clinical manifestations. The *iceA1* allele was detected in 66.7% of isolates from patients with erosive gastritis, while *iceA2* was identified in 57.1% of isolates from patients with enanthematous gastritis. The *iceA1* allele may be associated with a more severe form of gastritis because *iceA1*-positive strains produce more inflammation-inducing cytokines, such as interleukin-8, which are potent chemotactic factors that activate polymorphonuclear leukocytes that contribute to enhanced inflammatory responses (13, 30). This finding agrees with those of previous studies (24, 25). In this work, a statistically significant association was observed between *iceA* and the endoscopic diagnosis ($P = 0.047$).

TABLE 2. Distribution of the *cagA* gene and of the *vacA* and *iceA* alleles in isolates of *H. pylori* deriving from patients with different clinical manifestations

Genotype	% with clinical manifestation (no. affected/total no. of samples)	
	Enanthematous gastritis	Erosive gastritis
cagA genes[a]		
cagA positive	42.3 (11/26)	57.7 (15/26)
cagA negative	64.3 (9/14)	35.7 (5/14)
vacA genes[b]		
vacAs1a/m1	20.0 (1/5)	80.0 (4/5)
vacAs1b/m1	50.0 (4/8)	50.0 (4/8)
vacAs1a/m2	40.0 (2/5)	60.0 (3/5)
vacAs1b/m2	33.3 (2/6)	66.7 (4/6)
vacAs2/m2	80.0 (8/10)	20.0 (2/10)
vacAs1b/m1m2	66.7 (2/3)	33.3 (1/3)
vacAs2/m1m2	50.0 (1/2)	50.0 (1/2)
vacA negative		100.0 (1/1)
iceA genes[c]		
iceA1 + *iceA2*		100.0 (2/2)
iceA1	33.3 (3/9)	66.7 (6/9)
iceA2	57.1 (16/28)	42.9 (12/28)
iceA negative	100.0 (1/1)	

[a] $P = 0.185$.
[b] $P = 0.350$.
[c] $P = 0.047$.

Based on the data presented above, we conclude that the detection of *cagA*, *vacA*, and *iceA* genes allows an improved evaluation of the pathogenic potential from clinical isolates. In this study, the *cagA* gene, the combination *vacAs1a/m1*, and the *iceA1* allele were related to erosive gastritis; similarly, the combination *vacAs2/m2* and the *iceA2* allele were related to an attenuated form of gastritis. Therefore, the genotyping of the microorganism appears to be a clinically relevant procedure and can contribute to the prognosis of *H. pylori* infection.

REFERENCES

1. **Araya, J. C., L. Anabalón, I. Roa, M. Bravo, M. A. Villaseca, P. Guzmán, and J. C. Roa.** 2004. Relación de la genotipificación de *Helicobacter pylori* con la forma e intensidad de la gastritis en población adulta portadora de patología gástrica benigna. Rev. Med. Chil. **132:**1345–1354.
2. **Arents, N. L. A., A. A. Van Zwet, J. C. Thijs, A. M. D. Kooistra-Smid, K. R. Van Slochteren, J. E. Degener, J. H. Kleibeuker, and L.-J. Van Doorn.** 2001. The importance of *vac*A, *cag*A, and *ice*A genotypes of *Helicobacter pylori* infection in peptic ulcer disease and gastroesophageal reflux disease. Am. J. Gastroenterol. **96:**2603–2608.
3. **Atherton, J. C., P. Cao, R. M. Peek, Jr., M. K. R. Tummuru, M. J. Blaser, and T. L. Cover.** 1995. Mosaicism in vacuolating cytotoxin alleles of *Helicobacter pylori*: association of specific *vac*A types with cytotoxin production and peptic ulceration. J. Biol. Chem. **270:**17771–17777.
4. **Benenson, S., D. Halle, B. Rudensky, J. Faber, Y. Schlesinger, D. Branski, N. Rabinowitz, and M. Wilschanski.** 2002. *Helicobacter pylori* genotypes in Israeli children: the significance of geography. J. Pediatr. Gastroenterol. Nutr. **35:**680–684.
5. **Blaser, M. J., G. I. Perez-Perez, H. Kleanthous, T. L. Cover, R. M. Peek, P. H. Chyou, G. N. Stemmermann, and A. Nomura.** 1995. Infection with *Helicobacter pylori* strains possessing *cag*A is associated with an increased risk of developing adenocarcinoma of the stomach. Cancer Res. **55:**2111–2115.
6. **Bukanov, N. O., and D. E. Berg.** 1994. Ordered cosmid library and high-resolution physical-genetic map of Helicobacter pylori strain NCTC 11638. Mol. Microbiol. **11:**509–523.
7. **Caner, V., M. Yilmaz, N. Yonetci, S. Zencir, N. Karagenc, I. Kaleli, and H. Bagci.** 2007. *H. pylori ice*A alleles are disease-specific virulence factors. World J. Gastroenterol. **13:**2581–2585.
8. **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. U. S. A. **93:**14648–14653.
9. **Chiarini, A., C. Cala, C. Bonura, A. Gullo, G. Giuliana, S. Peralta, F. D'Arpa, and A. Giammanco.** 2009. Prevalence of virulence-associated genotypes of *Helicobacter pylori* and correlation with severity of gastric pathology in patients from western Sicily, Italy. Eur. J. Clin. Microbiol. **28:**437–446.
10. **Clayton, C. L., H. Kleanthous, P. J. Coates, D. D. Morgan, and S. Tabaqchali.** 1992. Sensitive detection of *Helicobacter pylori* by using polymerase chain reaction. J. Clin. Microbiol. **30:**192–200.
11. **Cover, T. L.** 1996. The vacuolating cytotoxin of *Helicobacter pylori*. Mol. Microbiol. **20:**241–246.
12. **Datta, S., S. Chattopadhyay, A. Chowdhury, A. Santra, D. R. Saha, T. Ramamurthy, S. K. Bhattacharya, D. E. Berg, G. B. Nair, and A. K. Mukhopadhyay.** 2005. Diagnosis and genotyping of *Helicobacter pylori* by polymerase chain reaction of bacterial DNA from gastric juice. J. Gastroenterol. Hepatol. **20:**1253–1259.

13. **Dunn, B. E., H. Cohen, and M. J. Blaser.** 1997. *Helicobacter pylori*. Clin. Microbiol. Rev. **10:**720–741.
14. **Fonseca, T. L., E. P. Moraes, C. R. Juliano, A. M. Silva, C. J. Scaini, R. A. Mendoza-Sassi, and P. E. A. Silva.** 2009. Detection of *Helicobacter pylori* by phenotypic and genotypic methods. Dig. Dis. Sci. **55:**1643–1648.
15. **Gatti, L. L., E. K. F. Souza, K. R. Leite, E. L. S. Bastos, L. R. Vicentini, L. C. Silva, M. A. C. Smith, and S. L. M. Payão.** 2005. *cag*A *vac*A alleles and *bab*A2 genotypes of *Helicobacter pylori* associated with gastric disease in Brazilian adult patients. Diagn. Microbiol. Infect. Dis. **51:**231–235.
16. **Gatti, L. L., J. L. P. Módena, S. L. M. Payão, M. A. C. Smith, Y. Fukuhara, J. L. P. Módena, R. B. Oliveira, and M. Brocchi.** 2006. Prevalence of Helicobacter pylori *cag*A, *ice*A and *babA2* alleles in Brazilian patients with upper gastrointestinal diseases. Acta Trop. **100:**232–240.
17. **Go, M. F.** 2002. Review article: natural history and epidemiology of *Helicobacter pylori* infection. Aliment. Pharmacol. Ther. **16:**3–15.
18. **Godoy, A. P. O., M. L. Ribeiro, Y. H. B. Benvengo, L. Vitiello, M. C. B. Miranda, S. Mendonça, and J. Pedrazzoli, Jr.** 2003. Analysis of antimicrobial susceptibility and virulence factors in *Helicobacter pylori* clinical isolates. BMC Gastroenterol. **3:**20.
19. **Kullavanijaya, P., D. Thong-Ngam, O. Hanvivatvong, P. Nunthapisud, P. Tangkijvanich, and P. Suwanagool.** 2004. Analysis of eight different methods for the detection of *Helicobacter pylori* infection in patients with dyspepsia. J. Gastroenterol. Hepatol. **19:**1392–1396.
20. **Kumar, S., A. Kumar, and V. K. Dixit.** 2008. Direct detection and analysis of *vacA* genotypes and *cagA* gene of *Helicobacter pylori* from gastric biopsies by a novel multiplex polymerase chain reaction assay. Diagn. Microbiol. Infect. Dis. **62:**366–373.
21. **Mattar, R., A. F. Santos, J. N. Eisig, T. N. Rodrigues, F. M. Silva, R. M. Lupinacci, K. Iriya, and F. J. Carrilho.** 2005. No correlation of *bab*A2 with *vac*A and *cag*A genotypes of *Helicobacter pylori* and grading of gastritis from peptic ulcer disease patients in Brazil. Helicobacter **10:**601–608.
22. **Mégraud, F., and P. Lehours.** 2007. *Helicobacter pylori* detection and antimicrobial susceptibility testing. Clin. Microbiol. Rev. **20:**280–322.
23. **Módena, J. L. P., A. I. L. Sales, G. O. Acrani, R. Russo, M. A. V. Ribeiro, Y. Fukuhara, W. D. Silveira, J. L. P. Módena, R. B. Oliveira, and M. Brocchi.** 2007. Association between *Helicobacter pylori* genotypes and gastric disorders in relation to the *cag* pathogenicity island. Diagn. Microbiol. Infect. Dis. **59:**7–16.
24. **Peek, R. M., Jr., S. A. Thompson, J. P. Donahue, K. T. Tham, J. C. Atherton, M. J. Blaser, and G. G. Miller.** 1998. Adherence to gastric epithelial cells induces expression of a *Helicobacter pylori gene*, *ice*A, that is associated with clinical outcome. Proc. Assoc. Am. Physicians **110:**531–544.
25. **Peek, R. M., Jr., L.-J. van Doorn, J. P. Donahue, K. T. Tham, C. Figueiredo, M. J. Blaser, and G. G. Miller.** 2000. Quantitative detection of *Helicobacter pylori* gene expression in vivo and relationship to gastric pathology. Infect. Immun. **68:**5488–5495.
26. **Ribeiro, M. L., A. P. O. Godoy, Y. H. B. Benvengo, S. Mendonca, and J. Pedrazzoli, Jr.** 2003. Clinical relevance of the *cag*A, *vac*A and *ice*A genotypes of *Helicobacter pylori* in Brazilian clinical isolates. FEMS Immunol. Med. Microbiol. **36:**181–185.
27. **Rota, C. A., J. C. Pereira-Lima, C. Blaya, and N. B. Nardi.** 2001. Consensus and variable region PCR analysis of *Helicobacter pylori* 3′ region of *cagA* gene in isolates from individuals with or without peptic ulcer. J. Clin. Microbiol. **39:**606–612.
28. **Van Doorn, L.-J., C. Figueiredo, R. Sanna, S. Pena, P. Midolo, E. K. W. Ng, J. C. Atherton, M. J. Blaser, and W. G. V. Quint.** 1998. Expanding allelic diversity of *Helicobacter pylori vacA*. J. Clin. Microbiol. **36:**2597–2603.
29. **Van Doorn, L.-J., C. Figuereido, R. Sanna, A. Plaisier, P. Schneeberger, W. de Boer, and W. Quint.** 1998. Clinical relevance of the *cag*A, *vac*A, and *ice*A status of *Helicobacter pylori*. Gastroenterology **115:**58–66.
30. **Xu, Q., and M. J. Blaser.** 2001. Promoters of the CATG-specific methyltransferase gene *hpyIM* differ between *iceA1* and *iceA2 Helicobacter pylori* strains. J. Bacteriol. **183:**3875–3884.
31. **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.