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Virulence Genes in *Helicobacter pylori* Strains from West Bengal Residents with Overt *H. pylori*-Associated Disease and Healthy Volunteers

Santanu Chattopadhyay,[1] Simanti Datta,[1] Abhijit Chowdhury,[2] Sujit Chowdhury,[2] Asish K. Mukhopadhyay,[3] K. Rajendran,[1] S. K. Bhattacharya,[1] Douglas E. Berg,[3] and G. Balakrish Nair[1,4]*

National Institute of Cholera and Enteric Diseases[1] and Department of Gastroenterology, Institute of Post Graduate Medical Education and Research,[2] Calcutta, India; Department of Molecular Microbiology, Washington University Medical School, St. Louis, Missouri 63110[3]; and International Centre for Diarrhoeal Diseases Research, Dhaka, Bangladesh[4]

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We compared putative molecular markers of virulence (*vacA*, *cagA*, and *iceA*) of *Helicobacter pylori* strains isolated from 52 adult duodenal ulcer patients from West Bengal, India, with those of *H. pylori* strains isolated from 48 adult healthy volunteers from the same region. On the basis of genotyping by PCR, we conclude that the *H. pylori* strains isolated from the two study groups were indistinguishable and that there are geographic variations in the association of certain putative *H. pylori* virulence genes with clinical status.

Helicobacter pylori infection is associated with duodenal ulcer (DU) or gastric ulcer, gastritis, and gastric adenocarcinoma (5). Although more than half of the population worldwide acquires *H. pylori* infection early in life, probably from their parents and older siblings (5), only about 10% suffer from overt disease, which mostly occurs in the later part of life, while large portions of the *H. pylori*-infected population remain asymptomatic carriers or have chronic gastritis (15). Therefore, it appears that both pathogenic and nonpathogenic strains of *H. pylori* coexist, as has been observed for several other bacteria, and those associated with disease may be endowed with a specific genotype (12).

Some strains of *H. pylori* can produce vacuoles in various cell lines due to the cytotoxic effect of the vacuolating cytotoxin, which comprises a signal sequence, a midregion, and a C-terminal end, coded by the *vacA* gene. The signal sequence in *vacA* is either s1 or s2, and the midregion of *vacA* can also be divided into m1 and m2. Strains carrying the s1ml mosaic combination of the *vacA* gene exhibit higher levels of cytotoxic activity than s1m2 strains, while s2m2 strains do not secrete the vacuolating cytotoxin (10). Furthermore, in Western countries, *H. pylori* strains which carry the s1m1 mosaic combination of *vacA* also usually carry a 40-kb pathogenicity island, whose marker gene is *cagA* (which encodes cytotoxin-associated gene product A). Strains that carry this pathogenicity island are associated with disease significantly more often than strains that do not carry this pathogenicity island (4). *iceA1*, an allele whose sequence has a high degree of homology to the sequence of *nlaIIIR* (which encodes a CATG-specific restriction endonuclease [*Nla*III] in *Neisseria lactamica*), is replaced by *iceA2* in some strains. The promoters of *hpyIM*, a CATG-specific methylase gene, which exists immediately downstream of *iceA*, may differ between *iceA1* and *iceA2* strains. It is not clear, however, how strains carrying *iceA1* can produce higher levels of the proinflammatory cytokine interleukin 8 in the gastric mucosa and are more often associated with DU in the West than strains carrying *iceA2* (17).

Most of the studies described above were performed with strains from symptomatic individuals, while the control groups in those studies comprised asymptomatic carriers (3, 6), which does not necessarily mean that they were healthy volunteers. To our knowledge very few studies that have compared *H. pylori* strains from patients with *H. pylori*-associated disease with strains from healthy volunteers have been described in the literature. In India, virtually no study on the genotypic status of *H. pylori* strains in relation to clinical outcome has been conducted except for our earlier study of the genotypes of strains isolated from DU patients and of DNA extracted from the gastric juice or from strains cultured from patients with gastritis (11). This lack of information was the impetus for this study.

Biopsy specimens were obtained as described earlier (11) from 52 adult Bengali patients of both sexes with a diagnosis of DU on the basis of endoscopic examination of the stomach and duodenum and 48 adult Bengali healthy volunteers (HVs) of both sexes who had no gastritis or dyspeptic syndromes like abdominal pain. The DU patients were recruited from patients seeking care for gastroduodenal disease at the Hospital of the Institute of Post Graduate Medical Education and Research in Calcutta, India, while the HVs were recruited on request from among a variety of individuals, including medical students and semiskilled and unskilled laborers. Written informed consent was obtained from all the individuals according to the recommendations of the Ethical Committee of the Institute of Post Graduate Medical Education and Research. One biopsy specimen from each patient was used for an in-house rapid urease test, while a second biopsy specimen was transported in 1 ml of brucella broth (Difco, Detroit, Mich.) containing 25% glycerol under cold conditions for culture at the *Helicobacter* unit of the National Institute of Cholera and Enteric Diseases. *H. pylori*

* Corresponding author. Mailing address: National Institute of Cholera and Enteric Diseases, P-33, CIT Rd., Scheme XM, Beliaghata, P.O. Box 177, Calcutta 700010, India. Phone: 91-33-350-4598. Fax: 91-33-350-5066. E-mail: gbnair@icddrb.org.

TABLE 1. Genotypes of *H. pylori* strains from patients with DUs and HVs

Genotype	No. (%) of strains from:	
	DU patients (n = 45)	HVs (n = 40)
s1	44 (97.8)	39 (97.5)
s2	1 (2.2)	1 (2.5)
m1	29 (64.4)	26 (65)
m2	16 (35.6)	14 (35)
cagA positive	43 (95.6)	38 (95)
cagA negative	2 (4.4)	2 (5)
iceA1	26 (57.8)	25 (62.5)
iceA2	15 (33.3)	13 (32.5)
iceA negative	4 (8.9)	2 (5)

strains were isolated on brain heart infusion (BHI; Difco) agar plates supplemented with 7% sheep blood, 0.4% IsoVitale X (BBL), and Dent (Oxoid, Basingstoke, England) in an atmosphere of 10% CO_2–5% O_2–85% N_2 in a double gas incubator (Heraeus Instruments, Hanau, Germany) and were identified on the basis of their typical morphologies and positivities by urease, oxidase, and catalase tests and by subsequent gene-specific tests. The *H. pylori* cells that grew from a biopsy specimen on the primary culture plate were collected as a pooled population and were preserved in sterile BHI broth with 20% glycerol at −70°C.

Chromosomal DNA from bacterial pellets was prepared from confluent growth on BHI agar plate cultures by the cetyltrimethylammonium bromide extraction method (2). PCRs for detection of the *vacA* and *iceA* alleles were carried out by using the primers and PCR conditions described previously (11). We used the primers described by Yang et al. (19) to amplify the 208-bp *cagA*-specific products, and the reaction was carried out in 25-μl reaction volume containing 10 ng of genomic DNA, 25 pmol of each primer, each deoxynucleoside triphosphate (Takara) at a concentration of 0.25 mM, 1 U of *Taq* DNA polymerase (Takara), and 1.5 mM $MgCl_2$ in a standard PCR buffer (Takara). All the products were amplified under the following conditions: 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min for 35 cycles in a Perkin-Elmer 9700 thermal cycler. Positive and negative controls were included in each assay.

All 100 biopsy specimens included in this study gave a positive result by the rapid urease test. On the basis of PCRs with specific primers for alternative alleles of *vacA* s1 and s2, *vacA* m1 and m2, and *iceA1* and *iceA2*, mixed genotype patterns were observed for isolates in cultures of specimens from 15 of the 100 subjects (7 were from DU patients and 8 were from HVs), indicating the prevalence of mixed infections. These isolates were eliminated from the final analysis. A total of 85 strains (45 from DU patients and 40 from HVs) whose genotypes did not indicate the presence of a mixed infection were further analyzed in this study.

We found that strains of *H. pylori* carrying the s1 allele of *vacA*, *cagA*, and *iceA1* predominate in West Bengal, India (Table 1). Previous descriptions of the vacuolating activities of strains with different *vacA* subtypes demonstrate that, in vitro, s1 strains secrete larger amounts of cytotoxin than s2 strains, which are known to be less virulent since they have a 12-amino-acid type s2 hydrophilic amino-terminal extension that results in the less efficient formation of membrane channels compared to the efficiency of membrane channel formation by s1 strains (9, 10). Recently, De Gusmao et al. (6) demonstrated a strong association between the presence of the s1 allele and the presence of DUs in Brazilian children. In contrast, our findings demonstrate the high frequency of occurrence of the s1 allele among adult HVs (Table 1). Therefore, there is a clear geographic difference in the prevalence of a particular genotype among patients with DUs and HVs.

De Gusmao et al. (6) also observed an association between the presence of the m1 allele of the *vacA* gene and the presence of peptic ulcers in children. The toxigenic role of m1 was established by experiments that showed that the product of *vacA* m1 can produce vacuoles, which are acidic in nature, in HeLa cells (1, 14). m2 strains, on the contrary, were thought to be the less toxigenic, as culture supernatants of m2 strains cannot produce vacuoles in HeLa cells, mainly because the toxin does not bind to the HeLa cell membrane (10). However, subsequent studies have shown that the product of the m2 allele also can produce vacuoles in RK13 cells (13). In our study, we have found that the profiles of the midregion of the *vacA* gene in strains isolated from DU patients and HVs were almost the same (Table 1).

In this study *cagA*-positive *H. pylori* strains were found to predominate (95.3%) over *cagA*-negative strains in West Bengal. This virulence marker was observed at almost equal frequencies in strains from DU patients and HVs (Table 1), indicating that the presence of the *cagA* gene cannot be considered a key virulence factor for determination of the clinical status of the host, as has been reported for strains from other geographic regions (16). Moreover, in this study we have found that *iceA1* and *iceA2* were almost equally distributed among strains from DU patients and HVs. This result demonstrates that, unlike in the West, *iceA1* is not associated with disease in West Bengal and is analogous to an earlier observation made in Japan (7).

In an attempt to find associations between the presence of the combination of the *cagA* gene and the *vacA* and *iceA* alleles and disease outcome by using *H. pylori* strains from different countries (Korea, Japan, the United States, and Colombia), Yamaoka et al. (18) have concluded that the presence of neither *iceA* nor particular combinations of *iceA*, *vacA*, and *cagA* was helpful in predicting a patient's disease status. From earlier studies, it is evident that the genotype varies among *H. pylori* strains isolated from different geographic regions (5, 8), which means that strains from patients with overt *H. pylori*-associated disease and from healthy individuals of the same geographic region should be evaluated for disease-specific genotypes. Importantly, toxin gene expression by *H. pylori* is inconsistent with the general phenomenon observed for several pathogenic bacteria, that is, that expression of key toxin genes is strictly associated with disease. Therefore, our aim was to find the most probable *H. pylori* genotype associated with disease and its distinctiveness from the genotypes of *H. pylori* strains isolated from healthy individuals in West Bengal. We found that s1m1 *cagA iceA1* was the dominant genotype in West Bengal and that the prevalence of this genotype was higher than those of the other allelic combinations in both the DU patient and HV populations (Fig. 1). Statistical analysis by the chi-square test and Fisher's exact test, with significance set at a P value of <0.05, further confirmed the absence of signif-

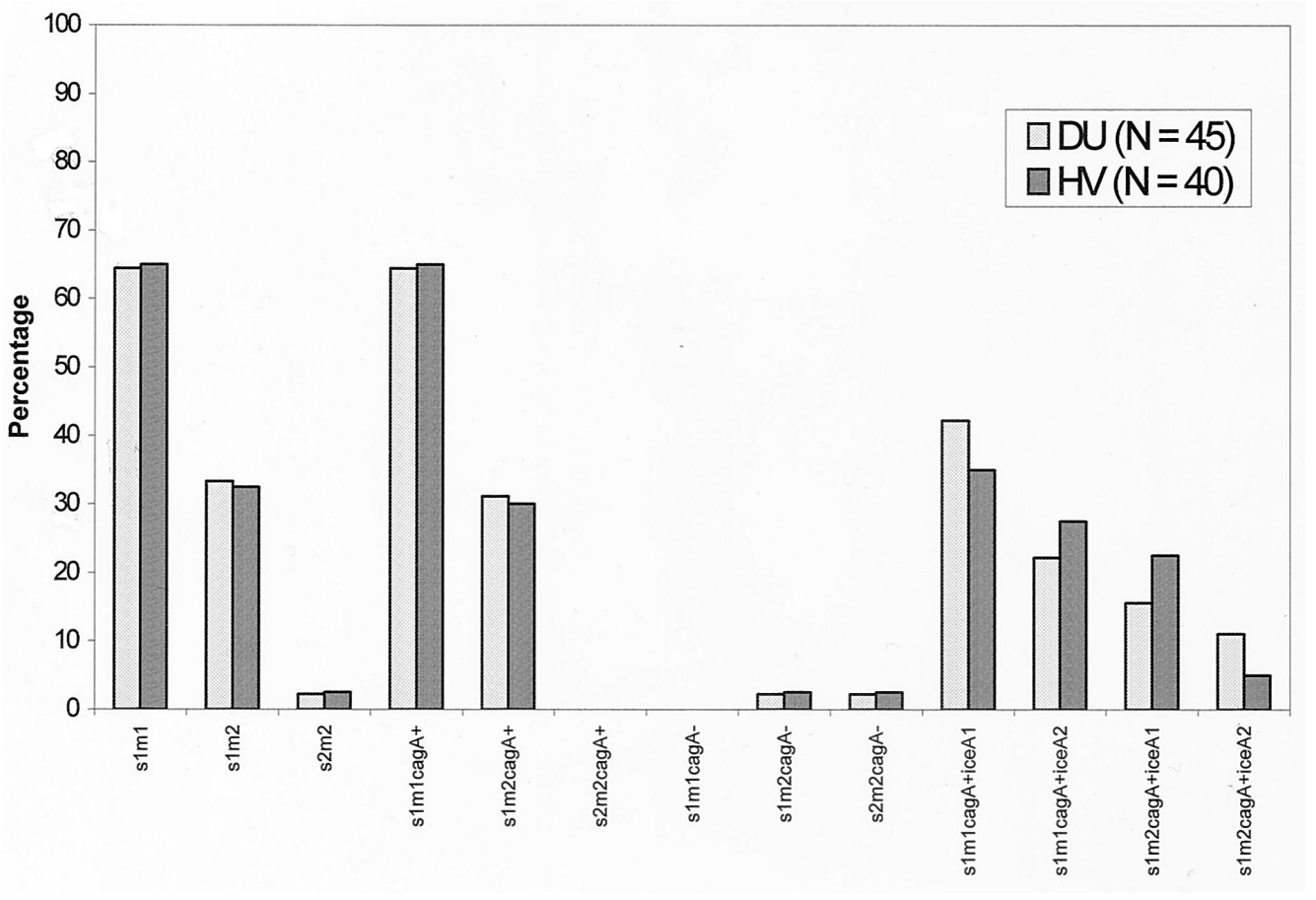

FIG. 1. Combinations of *vacA*, *cagA*, and *iceA* genotypes of *H. pylori* strains isolated from DU patients and HVs. +, positive for the gene; −, negative for the gene.

icant differences in the genotypes of strains isolated from DU patients and HVs (data not shown). We conclude that the strains isolated from the two study groups were almost indistinguishable and that no association could be made between the presence of these virulence marker genes or their combinations and the clinical status of *H. pylori*-infected individuals in West Bengal.

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