Equally High Prevalences of Infection with *cagA*-Positive *Helicobacter pylori* in Chinese Patients with Peptic Ulcer Disease and Those with Chronic Gastritis-Associated Dyspepsia

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Approximately 60% of *Helicobacter pylori* isolates in the Western world possess the cytotoxin-associated gene A (*cagA*). *cagA*-positive *H. pylori* is found to be associated with peptic ulcer disease (PUD) and gastric adenocarcinoma. To investigate the *cagA* status of *H. pylori* isolates from Chinese patients with PUD and chronic gastritis (CG), *H. pylori* populations from 83 patients, 48 with PUD and 35 with CG, were assessed by two different *cagA*-specific PCRs, Southern blotting, and colony hybridization. The combined results from PCR, Southern blotting, and colony hybridization indicate a prevalence of *cagA*-positive *H. pylori* isolates of 98% (47 of 48) among Chinese PUD patients and 100% (35 of 35) among Chinese CG patients. Amplification with primer sets 1 and 2 yielded 52 and 95% of the 82 *cagA*-positive Chinese *H. pylori*, respectively. In contrast, the sensitivity of *cagA*-specific PCR for *cagA*-positive *H. pylori* isolates from Dutch patients with primer set 1 was 92% (112 of 122) and that with primer set 2 was 91% (50 of 55). The prevalence of *cagA*-positive *H. pylori* populations in Chinese patients with PUD and CG is almost universally high. Therefore, *cagA* cannot be used as a marker for the presence of PUD in Chinese patients. Our data further suggest that allelic variation in *cagA* may exist and that distinct *H. pylori* genotypes may circulate in China and Western Europe.

Since the initial identification and successful culture of *Helicobacter pylori* from human gastric biopsy specimens by Marshall and Warren (11, 12), it has become accepted that this gram-negative spiral bacterium is a major cause of gastroduodenal diseases (7, 9, 25, 27). Although nearly all *H. pylori*-infected persons develop gastritis (8, 20), it remains unclear whether bacterial, host, or dietary factors determine the differences in the extent of the mucosal lesions induced by *H. pylori* among them. Only a proportion of *H. pylori*-colonized subjects develop peptic ulceration, and few of them have an increased risk of developing gastric cancer. Differences among bacterial strains may induce the divergent clinical or pathologic effects of *H. pylori* infection. Two phenotypic characteristics among *H. pylori* strains, the vacuolating cytotoxin (3) and the high-molecular-weight protein (6) encoded by the cytotoxin-associated gene A (*cagA*), have been found to be associated with distinct gastrointestinal disorders. The *cagA* gene has been cloned and its sequence has been determined (2, 23). Although the gene encoding the vacuolating toxin is present in nearly all strains, the activity of this cytotoxin is found in only 40 to 60% of the *H. pylori* strains (3, 26). A total of 40 to 60% of the *H. pylori* strains from patients with peptic ulcer disease (PUD) and 30% of the strains from patients with chronic gastritis are cytotoxin positive (21, 26). About 60 to 80% of *H. pylori* strains express the 120- to 140-kDa CagA product (1, 6). Various studies in the United States (3), Great Britain (6), Italy (2), and The Netherlands (26) have demonstrated a strong association between the presence of antibodies to CagA

and PUD. An association between infection with *cagA*-positive *H. pylori* and PUD was also observed (26). In contrast, only in 50 to 60% of patients with chronic superficial gastritis were antibodies to CagA found (3). However, the data about the correlation between *cagA* and the cytotoxicity of *H. pylori* and gastroduodenal disease are mainly obtained from the North American and Western European populations (4, 19, 22, 26).

China is one of the countries with a high prevalence of *H. pylori* infection and a high incidence of gastroduodenal diseases. In China about 40% of children have acquired infection with *H. pylori* by 10 years of age, and the prevalence rises with age to about 70% in those over 30 years old (18, 28). In a previous study that used the Western immunoblot technique to determine the antibody response to *H. pylori* in 167 Chinese patients with gastric cancer, PUD, or chronic gastritis (CG), we showed that 97% of them had antibodies against a 138-kDa protein band, most probably the CagA protein (17). However, the characteristics of the infecting *H. pylori* strains remained unknown and the nature of this protein could not be assessed. Therefore, in the study described in this report we determined the prevalence of *cagA*-positive *H. pylori* strains among Chinese patients in relation to the occurrence of PUD and CG. The results of a study about the vacuolating cytotoxic properties of these strains will be dealt with in another report.

MATERIALS AND METHODS

Patient population. Strains were obtained from 83 consecutive *H. pylori*-infected adults derived from a group of *H. pylori*-positive patients who had undergone gastroduodenoscopy in two hospitals of Shanghai, China, because of dyspeptic complaints. Strains from patients who had a history of gastric surgery, were receiving steroids, had an active infection requiring current antimicrobial therapy, had taken antimicrobial agents within 2 weeks prior to endoscopy, or had active gastrointestinal bleeding were not included in the study. The patient population consisted of 48 males and 35 females with a mean age of 41.0 ± 8.7 years (age range, 27 to 72 years). The patients were classified by endoscopic examination as having duodenal ulcers ($n = 32$), gastric ulcers ($n = 3$), both

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duodenal and gastric ulcers (*n* = 9), or no evidence of mucosal ulceration but with CG as diagnosed by histopathological examination (*n* = 35). Four patients had no ulceration at the time of endoscopy but had a history of endoscopically diagnosed duodenal ulcers; thus, 48 patients were considered to have PUD and 35 patients were considered to have CG.

Endoscopy, histopathology, and *H. pylori* cultures. During each endoscopic procedure, two antral and two corpus mucosal biopsy specimens were obtained by use of biopsy forceps, which were cleansed with a detergent, disinfected with 70% ethanol, and rinsed with sterile water after each examination. One antrum and one corpus biopsy specimen were placed in 2 ml of normal saline at 4°C and used for bacteriological culturing. The other two specimens were fixed in 10% formalin for histopathological examination. Bacteriological and histological assessments of the mucosal biopsy specimens were carried out as described previously (24). Cultures were prepared by smearing biopsy specimens on the surface of horse blood agar plates (7% defibrinated horse blood in Columbia agar base; Oxoid CM 331; Unipath, Basingstoke, England) and horse blood agar plates containing Skirrow supplement (Unipath). *H. pylori* organisms were identified on the basis of typical colony morphology, characteristic appearance on Gram staining, and positive urease, oxidase, and catalase tests. The *H. pylori* isolates that grew on the primary culture plates of each of the antrum and corpus biopsy specimens were collected with swabs to obtain separately *H. pylori* populations from the antrum and the corpus. Each swab was shaken in 1.8 ml of 8% glycerol peptone, and the suspension was stored at −70°C. This bacterial suspension is designated the frozen primary culture. For analysis, an aliquot of the frozen primary culture suspension was thawed and cultured on horse blood agar plates. The *H. pylori* populations isolated either from the antrum or the corpus biopsy specimen from the 83 patients were assessed for their *cagA* status.

Preparation of genomic DNA for PCR. After thawing of an aliquot of the frozen primary cultures, bacterial suspensions were inoculated on horse blood agar plates and cultured at 37°C for 3 days in a microaerophilic environment. The bacteria were harvested, and genomic DNA was extracted by using the Insta Gene Matrix (Bio-Rad Laboratories, Hercules, Calif.) according to the manufacturer's instructions.

PCR amplification of *cagA*. Two sets of primer were designed on the basis of the published sequence (2). Primer set 1 consists of primer caga1 (5′-GATAT AGCCA CTACC ACCAC CG-3′; positions 1249 to 1270) (26) and primer caga2 (5′-GGAAA TCTTT AATCT CAGTT CGG-3′; positions 1797 to 1819). Set 2 includes primer caga5 (5′-GGCAA TGGTG GTCCT GGAGC TAGGC-3′; positions 1495 to 1519) and primer caga2. These two primer sets were used in a standard PCR mixture to produce products calculated to be 570 and 324 bp, respectively. Briefly, 10 ng of DNA was used in a PCR mixture of 25 μl containing 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 3.0 mM MgCl$_2$, and 0.1 mg of bovine serum albumin per ml. The incubation conditions with primer set 1 were as follows: 40 cycles of 1 min at 95°C, 1 min at 55°C, and 1.5 min at 72°C and a final 5-min incubation at 72°C. When primer set 2 was used the number of cycles was 35 and the annealing temperature was 60°C. The PCR products were analyzed by horizontal agarose (1.5%) gel electrophoresis with ethidium bromide staining as described previously (15). Two Dutch *H. pylori* strains with laboratory numbers 348 and 628 were used as the positive and negative controls for *cagA*, respectively (26). Negative and positive control amplifications were performed in every experiment. The genomic DNA from the bacterial species *Helicobacter fennelliae* Sweden E6, *Campylobacter fetus* Sweden 7473, *Campylobacter coli* Lior 46, *Campylobacter jejuni* Lior 2, *Haemophilus influenzae* HI 864, *Neisseria meningitidis* McAZ 4027 and McB 902506 ET 26, *Borrelia burgdorferi* B31, and *Escherichia coli* ATCC 25922 were tested by PCR with either primer set to assess the specificity of the PCR amplification of *cagA*.

Southern blotting. To test the sensitivity and the specificity of PCR with primer set 2, all PCR-negative and 20 PCR-positive *H. pylori* populations were analyzed by Southern blotting. The genomic DNA of *H. pylori* was prepared as described previously (10). After digesting the DNA with *Hind*III, the fragments were separated by electrophoresis on a 0.7% agarose gel and transferred to Zeta-Probe Blotting Membranes (Bio-Rad Laboratories) as described by Sambrook et al. (21). The probe used to detect *cagA* was made by PCR amplification with primer set 2 (caga5-caga2). The PCR products obtained from four *H. pylori* populations were pooled and run on a 1% agarose gel. After electrophoresis, the 324-bp PCR product was excised from the gel and extracted from the agarose by using QiaeX (Qiagen GmbH, Hilden, Germany). The probe was labelled with digoxigenin (DIG)-dUTP by using the random primed labelling kit (Boehringer Mannheim GmbH, Mannheim, Germany). Hybridization conditions were as described previously (26), except that the hybridization temperature was 68°C. Probes were detected with anti-DIG antibodies conjugated to alkaline phosphatase and stained according to the instructions of the manufacturer.

Colony hybridization. An aliquot from each of the frozen primary culture suspensions yielding approximately 200 to 400 colonies was grown on horse blood agar plates for 3 days. Colonies from the plates were transferred onto a nylon membrane (Hybond-N; Amersham Life Science, Little Chalfont, England) by replica plating. The colonies were lysed and denatured, and then hybridization for *cagA* was done as described previously (26). The probe used to detect *cagA* in the colony hybridization assay was identical to that used in the Southern blotting mentioned above.
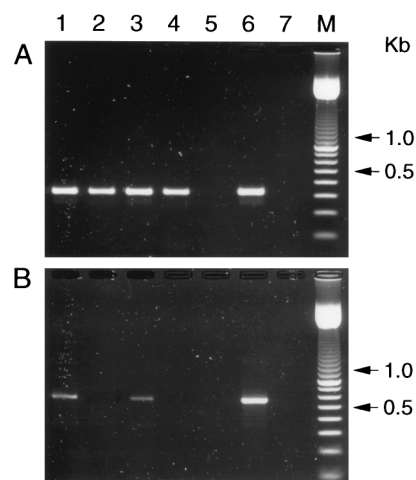

FIG. 1. Agarose gel (1.5%) electrophoresis with ethidium bromide staining of a 324-bp PCR amplicon of the *cagA* gene with primer set 2 (A) and a 570-bp PCR product with primer set 1 (B). Lanes 1 to 5, Chinese *H. pylori* isolates; lane 6 and lane 7, *cagA*-positive and *cagA*-negative *H. pylori* controls, respectively; lane M, 100-bp ladder.

Statistical analysis. Statistical analysis was performed by using the two-tailed Fisher's exact probability test. A *P* value of <0.05 was considered statistically significant.

RESULTS

Specificity of PCR assay. The specificity of PCR with either primer set 1 (caga1-caga2) or primer set 2 (caga5-caga2) targeting *cagA* was determined by testing bacterial strains, including *H. fennelliae*, various *Campylobacter* species, and bacterial species which are studied in our laboratory and which may occur as contaminants in our detection system. Only the *cagA*-positive control *H. pylori* strain amplified the expected 570- and 324-bp fragments in the *cagA* PCRs with primer set 1 and primer set 2, respectively. For the *cagA*-negative control *H. pylori* strain as well as any of the other bacterial species tested, the *cagA* PCR never yielded an amplification product (data not shown).

Sensitivity of *cagA*-specific PCR. The *cagA*-specific PCR with primer set 1 detected 112 of 122 *cagA*-positive *H. pylori* isolates from Dutch patients (26). So, the sensitivity of this PCR was 92%. This primer set was initially used to assess *cagA* in 10 *H. pylori* isolates from Chinese patients. Only 3 of the 10 isolates appeared to be *cagA* positive. However, when assessed by Southern blotting and colony hybridization, all 10 isolates appeared to be *cagA* positive. Therefore, primer set 2 was designed. Assessment of 55 *cagA*-positive *H. pylori* isolates from Dutch patients by this PCR (primer set 2) yielded *cagA* in 50 isolates (sensitivity, 91%). In order to determine the sensitivity of the PCR with these primers for Chinese strains, confirmation was done by Southern blotting and colony hybridization. PCR amplification yielded the expected 324-bp PCR product in 78 of 83 *H. pylori* from Chinese patients (Fig. 1). Four of these five *H. pylori* populations that were *cagA* negative by PCR were positive by Southern blotting and colony hybridization. In addition, 20 Chinese strains *cagA* positive by PCR were also positive by Southern blotting (Fig. 2). Furthermore, 30 *H. pylori* populations from Chinese patients *cagA* positive by PCR were also found to be *cagA* positive when assessed by colony hybridization. So, the sensitivity of this PCR with primer set 2 was 95% (78 of 82). In contrast, assessment of the 82 *cagA*-positive *H. pylori* isolates from Chinese patients by
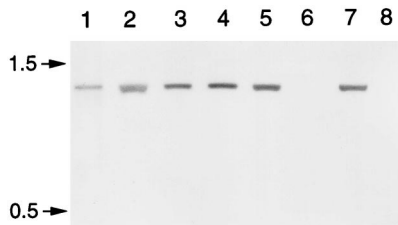
FIG. 2. Southern blotting of chromosomal DNAs from six Chinese *H. pylori* isolates (lanes 1 to 6, respectively), a *cagA*-positive *H. pylori* control (lane 7), and a *cagA*-negative *H. pylori* control (lane 8) digested with *Hin*dIII. The restriction fragments were separated on a 0.7% agarose gel. The DNAs were then transferred to a nylon membrane and hybridized with the *cagA* probe under conditions of high stringency. Numbers on the left are sizes (in kilobases).

PCR with primer set 1 yielded only 43 *cagA*-positive *H. pylori* strains (sensitivity, 52%). These results indicate sequence variation in the site complementary to primer caga1 in *cagA* of Dutch and Chinese *H. pylori* isolates.

Prevalence of *cagA* and relation with clinical outcome. The combined results from PCR, Southern blotting, and colony hybridization indicated an overall prevalence of *cagA*-positive *H. pylori* isolates of 99% (82 of 83) from Chinese patients with PUD or CG. *cagA* was detected in 47 of 48 (98%) *H. pylori* populations isolated from patients with current or previous PUD (of whom 36 had duodenal ulcer, 3 had gastric ulcer, and 9 had both duodenal and gastric ulcers) and in 35 (100%) *H. pylori* populations isolated from patients with CG. There was no significant difference between the prevalence of *cagA*-positive *H. pylori* isolates from the subgroups of patients with distinct clinical manifestations ($P = 1.0$).

DISCUSSION

The aim of this study was to determine the prevalence of *cagA* among *H. pylori* isolates and its relationship with PUD and CG in 83 Chinese patients. The specificity of the *cagA* PCR was certified by using DNAs from a *cagA*-positive and a *cagA*-negative control *H. pylori* strain and various other bacterial species as templates in the PCR amplification. By PCR with primer set 2, our results indicated that 78 Chinese *H. pylori* populations possessed *cagA*. Four of five strains *cagA* negative by PCR were found to be positive when assessed by Southern blotting and colony hybridization with the *cagA*-specific probe under stringent conditions. In addition, 20 *H. pylori* strains positive for *cagA* by PCR were confirmed to be positive by Southern blotting and 30 *H. pylori* strains positive for *cagA* by PCR were confirmed to be positive by colony hybridization. The expression of the CagA protein was also assessed in 20 *cagA*-positive and 1 *cagA*-negative *H. pylori* populations by the Western blotting (immunoblotting) technique with anti-CagA-positive serum. The results indicated that the CagA protein was expressed by the 20 *cagA*-positive *H. pylori* strains tested and not by the *cagA*-negative *H. pylori* strain (data not shown), being consistent with the results obtained by PCR, Southern blotting, and colony hybridization. Together, by using these methods, 82 of 83 (99%) *H. pylori* strains were *cagA* positive.

This high prevalence of *cagA*-positive *H. pylori* (99%) among Chinese patients with PUD and CG is almost completely consistent with our previous study in which 97% of serum samples from 167 Chinese patients with CG, PUD, and gastric cancer had antibodies against a 138-kDa protein, most probably CagA (17). In previous studies with populations from Europe and the United States, the prevalence of *cagA*-positive *H. pylori* varied

between 69 and 79% (4, 16, 26). Thus, the prevalence of *cagA*-positive *H. pylori* among Chinese patients with PUD or CG is much higher than that among North American and European *H. pylori*-infected patients. The only other study regarding a population outside Europe and the United States showed a similarly high prevalence of *cagA*-positive *H. pylori* isolates among *H. pylori*-infected patients (13). Recently, Miehlke et al. (13) found that *cagA* could be identified in 98% (59 of 60) of *H. pylori* isolates from Korean patients with duodenal ulcer and gastric carcinoma. On the other hand, the prevalence of *cagA*-positive *H. pylori* isolates from 16 patients of Moroccan ethnicity (most of whom emigrated to Belgium 20 to 30 years ago and who are currently living there) was significantly lower (31.3%) than that among 57 Belgian patients (73.6%) or 23 non-Belgian, non-Moroccan patients (82.6%) (4). One possible explanation for the difference between the prevalence of *cagA* may be that a specific genotype may exist within a particular geographic area or within a particular ethnic group.

The association between *cagA*-positive *H. pylori* and clinical gastrointestinal disorders, especially PUD, has been shown in a number of studies. Using combined serological and genetic detection methods, Cover et al. (4) found a strong correlation between the presence of antibodies to CagA in serum and the isolation of a *cagA*-positive strain from a patient. In addition, the prevalence of *cagA*-positive *H. pylori* was higher among patients with duodenal ulcer disease than among patients with nonulcer dyspepsia. Evidence of the association of *cagA*-positive *H. pylori* with peptic ulceration has been provided by reverse transcription PCR applied to stomach biopsy specimens (19). Recently, studying 155 *H. pylori*-infected Dutch patients, we found that the occurrence of *cagA*-positive *H. pylori* among 76 (93%) patients with PUD was higher than that among 79 (65%) patients with functional dyspepsia, also indicating that *cagA* is associated with PUD (26). However, our present study together with previous results (17) indicated that almost all of the *H. pylori* populations (99%) isolated from Chinese patients possessed *cagA*, whereas the prevalence of *cagA*-positive *H. pylori* isolates from patients with PUD and CG was similar. Therefore, *cagA* cannot be used as a marker for PUD in *H. pylori*-infected Chinese patients. This is consistent with a recent report by Mitchell et al. (14), in which the seroprevalence of anti-CagA antibodies among Chinese *H. pylori*-infected gastric cancer patients and *H. pylori*-infected asymptomatic subjects was examined by Western blotting. An equally high prevalence of antibodies to CagA in both groups (83 versus 86%) was observed. On the other hand, because *cagA* and its product, the 128- to 140-kDa protein, exist in almost all *H. pylori* populations from Chinese patients with symptoms of dyspepsia and because of its strong immunogenicity, the CagA protein may be used as an antigen for the serodiagnosis of *H. pylori* infection and the candidate antigen for immunization against *H. pylori* in Chinese patients instead of being a marker for PUD.

When primer set 1 (caga1-caga2) was used to detect *cagA* by PCR amplification, the expected 570-bp product was observed in only 52% of *cagA*-positive *H. pylori* populations from Chinese patients. In contrast, 92% (112 of 122) of the *cagA*-positive *H. pylori* populations were *cagA* positive by PCR amplification with this primer set when a collection of 155 *H. pylori* populations from Dutch patients with PUD and functional dyspepsia was assessed (26). Assessment of the prevalence of *cagA* among 55 Dutch *cagA*-positive *H. pylori* populations by PCR with primer set 2 yielded an equally high sensitivity (91%; 50 of 55). The difference in the detection between *H. pylori* populations from Chinese patients and those from Dutch pa-

tients with primer set 1 was highly significant ($P < 0.001$). These results indicate that the sequence of *cagA* differs between Dutch and Chinese *H. pylori* isolates, at least at the site complementary to primer caga1. However, sequencing of *cagA* is necessary for full elucidation of this observation. If it is true and if it is confirmed, it would indicate that allelic variation of the *cagA* gene of *H. pylori* strains from Chinese patients and *H. pylori* strains from Dutch patients has occurred.

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