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Intact *cag* pathogenicity island of *Helicobacter pylori* without disease association in Kolkata, India

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Abstract

Several genes including the *cagA* in the *cag* pathogenicity island (*cag* PAI) of *Helicobacter pylori* are thought to be associated with the gastroduodenal diseases and hence variation in the genetic structure of the *cag* PAI might be responsible for different clinical outcomes. Our study was undertaken to characterize the *cag* PAI of *H. pylori* strains from duodenal ulcer (DU) patients and asymptomatic or non-ulcer dyspepsia (NUD/AV) subjects from Kolkata, India. Strains isolated from 52 individuals (30 DU and 22 NUD/AV) were analyzed by PCR using 83 different primers for the entire *cag* PAI and also by dot-blot hybridization. Unlike *H. pylori* strains isolated from other parts of India, 82.6% of the strains used in this study had intact *cag* PAI, 9.6% had partially deleted *cag* PAI, and 7.7% of the strains lacked the entire *cag* PAI. Dot-blot hybridization yielded positive signals in 100% and 93.8% of PCR-negative strains for HP0522-523 and HP0532-HP0534 genes, respectively. An intact *cagA* promoter region was also detected in all *cagA*-positive strains. Furthermore, the expression of *cagA* mRNA was confirmed by RT-PCR for the representative strains from both DU and NUD/AV subjects indicating the active *cagA* promoter regions of these strains. A total of 66.7% of Kolkata strains produced a ~390-bp shorter amplicon than the standard strain 26695 for the HP0527 gene, homologue of virB10. However, sequence analyses confirmed that the deletion did not alter the reading frame of the gene, and mRNA transcripts were detected by RT-PCR analysis. The strains isolated from DU and NUD/AV express CagA protein and possess a functional type IV secretion system, as revealed by Western blot analyses. Interestingly, no significant differences in *cag* PAI genetic structure were found between DU and NUD/AV individuals suggesting that other bacterial virulence factors, host susceptibility, and environmental determinants also influence the disease outcome at least in certain geographical locations.

Keywords

Helicobacter pylori; *cag* PAI; Duodenal ulcer; Disease association

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Introduction

Helicobacter pylori, a Gram-negative microaerophilic bacterium, chronically infects the gastric epithelium, and infection is associated with several gastroduodenal diseases such as chronic gastritis, peptic ulcer, and gastric cancer (Correa et al., 1992; NIH, 1994; Parsonnet, 1999). Although more than half of the human population carries the infection, only ~10–15% of the infected individuals develop such gastroduodenal diseases and hence, strain-specific genetic traits could be involved in *H. pylori*-related pathogenesis. The *cagA* gene, which encodes a protein of ~128 kDa (CagA), the CagA protein is one of the most well-studied virulence markers of *H. pylori* (Covacci et al., 1993; Tummuru et al., 1993). *cagA* along with several other virulence-associated genes constitute the ~40-kb *cag* pathogenicity island (*cag* PAI) and is present in ~50–70% and ~90% of the western and Asian *H. pylori* strains, respectively (Covacci et al., 1993; Ito et al., 1997; van Doorn et al., 1999). Vacuolating cytotoxin (VacA), a protein that can cause severe cytotoxicity in cell lines as well as in gastric mucosa, is coded by the *vacA* gene, which could be present in several allelic combinations (Cover et al., 1994; Atherton et al., 1995). *H. pylori* strains that carry s1m1-allelic combination are significantly more cytotoxic than strains that carry s1m2-allelic combination while strains that carry s2m2-allelic combination are a non-vacuolating form of VacA (Atherton et al., 1995, 1997). Interestingly, strains that carry *cag* PAI (cag^+) are more likely to carry the s1m1-allelic combination of the *vac*A gene as compared to strains that lack *cag* PAI (cag^-) (Atherton et al., 1995, 1997). In western countries, strains that carry s1m1*cagA* are significantly associated with *H. pylori*-related gastroduodenal diseases, although such association is not apparent in the Indian context (Cover et al., 1994; Atherton et al., 1997; Mukhopadhyay et al., 2000; Chattopadhyay et al., 2002; Datta et al., 2003). Moreover, expression of *bab*A gene product, which governs adherence to $Lewis^b$ (histo-blood group antigen) on gastric epithelial cells and expression of Lewis antigens as part of their lipopolysaccharide, is also strongly associated with cag^+ strains (Ilver et al., 1998). Therefore, it appears that cag^+ and cag^- strains probably have different requirements for their colonization in gastric epithelium.

The *cag* PAI, which contains a different GC content than the *H. pylori* genome, probably entered the genome after the bacterium had evolved as a species (Tomb et al., 1998). It contains 27 genes, 6 of which are thought to encode a putative type IV secretion system, responsible for the translocation of the CagA into the host cell (Covacci et al., 1999; Stein et al., 2002). The CagA, after being translocated to the host cell, becomes phosphorylated on tyrosine residues by Src family kinases, and the phosphorylated CagA interacts with the SH2 domain of the SHP-2 (Higashi et al., 2002a, 2002b). This interaction leads to an altered cellular morphology and may eventually lead to gastric carcinoma (Asahi et al., 2000; Segal et al., 1999; Odenbreit et al., 2000; Stein et al., 2000; Higashi et al., 2002a).

An intact *cag* PAI may be responsible for the proinflammatory nature of *H. pylori* leading to gastroduodenal diseases like duodenal ulcer, gastric atrophy, and gastric cancer. The presence of intact *cag* PAI strains was found more frequently in patients with severe gastroduodenal disease (Nilsson et al., 2003). Partial deletions of the *cag* PAI appear to be sufficient to render the organism less pathogenic (Ali et al., 2005; Nilsson et al., 2003). The *cag* PAI is involved in the induction of interleukin-8 (IL-8) secretion, which is implicated in the inflammatory response of the gastric mucosa to *H. pylori* infection. However, the existence of strains inducing IL-8 secretion regardless of the *cag* PAI structure suggests that this region is not the only prerequisite for the IL-8 secretion (Audibert et al., 2001; Hsu et al., 2002). Furthermore, the *cag* PAI status did not affect the attachment of the bacterium to the gastric epithelial cells. In some populations, *cagA*-related genes are associated with an abrogated apoptotic response, whereas other studies showed that apoptosis was increased in

the antrum and body (of the stomach) only in patients with *cagA*-positive *H. pylori* strains (Peek et al., 1997; Moss et al., 2001). Moreover, *cag* PAI-positive *H. pylori* strains induce apoptosis more rapidly than *cag* PAI-negative mutant strains, suggesting that the *H. pylori* binding and subsequent apoptosis are differentially regulated with regard to bacterial properties (Minohara et al., 2007). The *cagA* sequences of *H. pylori* strains isolated from Kolkata, India, are clustered with the *cagA* sequences of strains isolated from western countries and differed significantly from *cagA* sequences of strains isolated from East Asia (China or Japan) (Mukhopadhyay et al., 2000; Datta et al., 2003; Chattopadhyay et al., 2004). There is also a distinct polymorphic site at the right end of the *cag* PAI of Kolkata *H. pylori* strains (Kersulyte et al., 2000). It has also been observed that the presence of the IS605 element both in $cagA^+$ and $cagA^-$ strains did not systematically modify the severity of associated disease in the study population (Owen et al., 2001).

Studies concerning the variation within the *cag* PAI of *H. pylori* infection associated with a variety of outcomes ranging from seemingly asymptomatic coexistence to peptic ulcer disease and gastric cancer showed variable results in different geographical populations (Yakoob, 2009). One study from southern India reported that intact *cag* PAI is present in only 12% of the population which correlated well with the data that 15% of the infected patients are symptomatic (Kauser et al., 2004). Another study claimed that the presence of an intact *cag* PAI correlated with the development of more severe pathology, and such strains were found more frequently in patients with severe gastroduodenal disease (Ali et al., 2005).

These considerations and our interest in the dynamics of genetic traits associated with *H. pylori* infection and disease association motivated us to conduct the present study (i) whether the Bengali population, which is different from the south Indian population, has a similarly low percentage of intact *cag* PAI carrying *H. pylori* strains, and (ii) whether the presence of intact *cag* PAI is correlated with the development of a more severe pathology to understand the disease process and pathogen–host interaction.

Materials and methods

Patient samples

A total of 73 adult participants [duodenal ulcer (DU) patients and non-ulcer dyspepsia (NUD) or asymptomatic volunteers (AV)] of both sexes (aged between 20 and 65 years) underwent a non-sedated upper gastrointestinal endoscopy (GIF XQ 30, Olympus optical company, Japan) under topical lignocaine anesthesia at the hospital of the Institute of Post Graduate Medical Education and Research, Kolkata, India, during the years 2002–2004. Among the 40 DU cases (17 females and 23 males), the mean age difference was 46±11.7 years vs. 43.7±9.2 years and among 33 NUD or AV cases (10 females and 23 males), the mean age difference was 31.4±6.5 years vs. 33.4±6.7 years, respectively. Diagnosis of duodenal ulcer was based on visual mucosal examination of the stomach and duodenum during endoscopy and also on any patient history of previous peptic ulcer. A detailed history was taken, and a physical examination of each subject was carried out prior to endoscopy. The objective of the study was explained to every individual. Informed consents were obtained from each individual under protocols approved by the institutional ethical committees of the Post Graduate Medical Education and Research and National Institute of Cholera and Enteric Diseases, Kolkata, West Bengal, India. Exclusion criteria were: use of antibiotics, antihistamins, and proton pump inhibitors 3 months prior to this study. During endoscopy, 2 biopsies, one from the antrum and the other from the corpus of the stomach, were obtained from each subject. Biopsies taken in 0.6 ml of Brucella broth (Difco Laboratories, Detroit, MI) with 15% glycerol were transported to the Bacteriology Division

of the National Institute of Cholera and Enteric Diseases in ice-cold condition and were stored at − 70°C until culture.

H. pylori culture

In the laboratory, Brucella broth containing the specimen was vortexed for 2 min and 200 µl of the mixture was streaked on Petri plates containing brain heart infusion (BHI) agar (Difco Laboratories) supplemented with 7% sheep blood, 0.4% IsoVitaleX, amphotericin B (8 µg/ml) (Sigma Chemicals Co., St. Louis, MO), trimethoprim (5 µg/ml) (Sigma Chemicals Co.), and vancomycin (Sigma Chemicals Co.) (6 µg/ml). Plates were incubated at 37°C in a double gas incubator (Heraeus Instruments, Germany), which maintains an atmosphere of 5% O_2, 10% CO_2, and 85% N_2, for 3–6 days. *H. pylori* colonies, which appeared as translucent water droplets, were identified based on their typical colony morphology and positive urease, oxidase, and catalase tests. The *H. pylori* cells were preserved in sterile BHI broth with 20% glycerol at −70°C.

Characterization by PCR-based assay

H. pylori genomic DNA was extracted by the CTAB (hexadecyltrimethyl ammonium bromide) method (Ausubel et al., 1993) from 24-h grown confluent lawn of bacterial culture on brain heart infusion agar (BHIA; Difco Laboratories) plates. Specific PCR was carried out in 20-µl volume containing 10 ng of bacterial genomic DNA, 20 pM of each primer, 0.25 mM of each dNTP (Takara, Shuzo, Japan), 1 U of Taq DNA polymerase (Takara) in standard PCR buffer (Takara) containing 1.5 mM $MgCl_2$, and the products were amplified in 35 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min (1 min/kb). Primers used for PCR, generating probes for hybridization, and nucleotide sequencing are listed in Table 1a and 1b. All strains underwent multiple PCR assays using 83 primers in various combinations. The strains were first amplified with Primer set A, and those strains which failed to amplify with the standard primer set A for a gene were further amplified by PCR using primers within and outside the gene designated as either primer set B or primer set C or both as shown in Table 2.

Analysis by dot-blot hybridization and nucleotide sequencing

PCR products were purified with the QIA quick gel extraction kit (Qiagen Corporation, Chatsworth, CA) according to the manufacturer's instruction and were directly sequenced using the BigDye terminator cycle sequencing kit (Perkin-Elmer, Applied Biosystems, Foster City, CA) on an automated DNA sequencer (ABI Prism 310). DNA sequence editing and analysis were performed with programs in the GCG package programs (Genetics Computer Group, Madison, WI). Dot-blot hybridization was performed by using Hybond-N1 nylon membranes and an ECL kit (Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturer's instructions. Probes for various genes were generated by PCR using *H. pylori* strain 26695 genomic DNA as the template with specific primer pairs.

Gene expression assay by semiquantitative RT-PCR

Total RNA was extracted from *H. pylori* culture using the RNeasy Mini kit (Qiagen) according to the manufacturer's protocol. RNA samples were electrophoresed in agarose gel, and the ethidium bromide-stained gel was inspected to check RNA quality. The absence of genomic DNA contamination was verified by PCR using *taq* DNA polymerase without reverse transcriptase. A total of 300 ng of RNA was reversely transcribed using the One-Step RTPCR Kit (Qiagen). The primers 5'- ACTCTAACGATCAAGAGATTATCAAAGG -3' (sense) and 5' - TGTATAAGGTTCTATTGGGATCGTCATT -3' (antisense) were used to amplify 288 bp of *cag*Y mRNA and Cag 5cf-Cag 3cr (Table 1b) for 350 bp of *cagA* mRNA. *Ure B* was amplified using the primers 5'- CGT CCG GCA ATA GCT GCC ATA

GT -3' (sense) and 5'- GTA GGT CCT GCT ACT GAA GCC TTA -3' (antisense) to generate a 464-bp product. Data for *cagY* mRNA were normalized to data for urease. The results were analyzed with Quantity One software (Bio-Rad).

Infection of AGS cells with *H. pylori* strains and immunoblotting

Human gastric epithelial AGS cells were cultured in RPMI 1640 (GIBCO BRL, Grand Island, NY) containing 10% fetal bovine serum (Invitrogen Corp., USA) at 37°C in a 5% CO_2–air humidified atmosphere. *H. pylori* (3×10^8 cells) was added to AGS cells (3×10^6 cells per 100-mm dish), which were then cultured in an antibiotic-free medium at a multiplicity of infection of 100. After incubation in a 5% CO_2 atmosphere for 5 h, infected AGS cells were washed 3 times with 0.01 M phosphate buffered saline (PBS) (pH 7.5) containing 2 mM Na_3VO_4 and then lysed in ice-cold lysis buffer containing 50 mM Tris, pH 6.8, NP 40, 10% glycerol, 5 mM EDTA (pH 8.0), 25 mM NaF, 2 mM sodium orthovanadate, 5 mM sodium pyrophosphate, 200 mM NaCl, and protease inhibitor cocktail (Roche). The cell lysates were centrifuged at 5000 × g for 10 min at 4°C, and protein estimation was done.

Equal amounts of cell lysate were separated by SDS-polyacrylamide gel electrophoresis (7.5% polyacrylamide) and blotted onto a nitrocellulose membrane (Transblot, Bio-Rad Laboratories, USA) at 20 V for 0.5 h at room temperature in transfer buffer. The membranes were blocked with 3% (w/v) bovine serum albumin in T-PBS [10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.5% Tween 20] for 2 h at 37°C under gentle shaking. After washing 3 times with TBST, each for 15 min, nitrocellulose strips were incubated with a primary antibody in T-PBS. After the membranes had been washed with T-TBS, they were incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase in T-TBS and visualized with an enhanced chemiluminescence detection system as directed by the manufacturer (Amersham Pharmacia Biotech. Inc.).

Antibodies

The primary antibody used for immunoblotting was an anti-CagA polyclonal antibody (Austral Biologicals, San Ramon, CA).

Nucleotide sequence accession numbers

The sequences obtained here were deposited in GenBank under accession numbers GQ396658-GQ396660.

Statistical analysis

Statistical analysis was done using Microsoft Excel 2003 (Microsoft Corporation, Redmont, USA) software.

Results

Among 73 individuals included in the study, 40 had clinical features of DU and 33 were NUD or asymptomatic subjects. In 30 out of 40 (75%) DU and 22 out of 33 (67%) NUD or asymptomatic subjects, evidence of *H. pylori* infection was confirmed from the isolation of this bacterium by culture method, and these were included in the further study.

Characterization of *cag* pathogenicity island (*cag* PAI) of *H. pylori*

In this study, *H. pylori* strains from 52 clinical isolates [DU (n=30), NUD/AV (n=22)] were analyzed. Of the 52 strains, 92.3% (n=48) were *cag* PAI-positive and the rest 7.7% (n=4) lacked the entire *cag* PAI genes. The *cag* PAI positivity includes both intact *cag* PAI and

partially deleted *cag* PAI. The *cag* PAI was defined as intact if all the gene sets of the *cag* PAI were present, whilst negative *cag* PAI was defined if none of the gene sets were present and gave a 550-bp amplicon with empty-site PCR. Partially deleted *cag* PAI was defined where a few, but not the whole set of *cag* PAI genes were present (Ikenoue et al., 2001). In our study using both PCR and dot-blot hybridization (DBH) methods, we found that 43 (82.6%) of the 52 strains had intact *cag* PAI, and 5 (9.6%) strains had partially deleted *cag* PAI (Table 2 and Figs. 1 and 2). In the *cag* PAI empty-site PCR where primers were designed from the flanking region of the *cag* PAI, 4 strains gave an amplicon of 550 bp indicating a complete lack of the *cag* PAI. Of the 5 partially deleted strains, 3 – I-110 (HP0535-HP0536), I-338 (HP0530-HP0531), and OSC36B (HP0532-HP0534) – had deletion in 2 genes, confirmed by both DBH and PCR assays, while the remaining 2 strains had deletions in multiple genes, and one further strain was devoid of the entire left end region of the *cag* PAI (data not shown).

PCR using multiple primer sets and dot-blot hybridization analysis

Analysis of the HP0519-521 regions showed that 10% of the strains produced around 150-bp higher amplicon (1414 bp) than the standard strain 26695 (1264 bp) using primers PAI-1S and PAI-1AS (Fig. 3A). After first screening by PCR, it was found that few primer pairs of the initial sets of primers (primer set A) failed to provide any amplicon in most of the Kolkata strains analyzed in this study (Fig. 1). These strains were subjected to DBH to evaluate whether the ORFs were truly absent or were falsely negative by PCR. It was found that most strains that were detected negative for a gene by PCR using primers from primer set A for a segment of a gene, hybridized with the corresponding probe in dot blot. For example, during analysis of HP0522-523 regions, 16 out of 48 *cag* PAI-containing strains were negative by PCR using primer sets A and B (Fig. 3B), but all (100%) PCR-negative strains showed positive signals after DBH with the specific probe generated by the same primers from 26695. Later on, primers designed from HP0519 (PAI-1S) and HP0524 (PAI-6AS) yielded around 4.8 amplicon size which is same as that of 26695 from the earlier PCR-negative strains indicating that these strains possessed the HP0522-523 regions (Fig. 3C). Similarly, for HP0532-HP0534 gene, 45 strains were negative by PCR with primer set PAI new2F-PAI 25AS (Fig. 4A) but after DBH with the specific probe generated by the same primer set from 26695, 45 out of 48 *cag* PAI-positive strains yielded positive signals (Fig. 4B). Those genes, which were not amplified by PCR with the primer set A, were successfully amplified with the second or the third sets of primers or by both sets (primer sets B and C) as given in Table 2. It is evident that using only primer set A, a mean of 29% (range 6.4–93.8%) of the genes could not be detected. Of these 29% undetected genes, primer sets B and C could detect *cag* PAI genes in a mean of 84% (range 38.5–100%) cases. Thus, the sensitivity of all the the primer sets A, B, and C in combination was 95% (Table 2).

cagY gene (HP0527) of *H. pylori*

CagY protein, homologue of virB10, is an outer membrane protein and plays an important role in the *cag* PAI as it encodes for type IV export machinery. To understand the distribution of the gene encoding this CagY protein among the *cag* PAI-positive strains, primers (PAI14S and PAI15AS) were designed from HP0527 and HP0528 genes of 26695. PCR assay using these 2 primers was able to amplify 46 strains (95.8%) among the 48 *cag* PAI-containing strains. Interestingly, 32 of 48 (66.7%) strains produced around 400-bp shorter (1.1 kb) amplicon size than the standard 26695 strain while 14 of 48 strains (29.1%) gave an amplicon same as that of the strain 26695 (1.5 kb) (Fig. 5A and 5B). Sequence analysis of the shorter sequences revealed that the strains having a shorter amplicon had a 390-bp deletion, although this deletion did not alter the reading frame (Fig. 5C). RT-PCR analysis revealed that the number of repeats at the 5’ end of HP0527 does not have any

effect on the level of mRNA transcription (Fig. 6). Interestingly, one strain showed no mRNA transcript for *cagY* while others showed various levels of transcription (lane 3, Fig. 6).

HP0535 gene of *cag* PAI

With primer pair PAI27S and PAI27AS designed from HP0535 and HP0536 gene of 26695, only 16 out of 48 *cag* PAI-positive strains gave a positive amplicon. All the strains, which did not amplify with PAI27S and PAI27AS, were evaluated with 2 more internal primers. These 2 primer sets, PAI27S-HP0535AS2 and HP0535S2-PAI27AS (Table 1a and 1b), were able to amplify all except 2 strains (93.5%, 29/31). However, among 46 strains that amplified, 31 gave a higher amplicon as shown in Fig. 7A and 7B.

The *cagA* gene and *cagA* promoter region and LEC of the *cag* PAI

The primers *cag*5cf and *cag*3cr were designed from conserved regions to amplify 350 bp of the *cagA* gene (Chattopadhyay et al., 2004). Fourty-eight strains gave a 350-bp amplicon with *cag*5cf and *cag*3cr primers showing that all the *cag* PAI-positive strains were *cagA*-positive. All the *cagA*-positive strains were positive for the ORF located in the extreme left of the *cag* PAI, namely HP0520, -521, and -522 (annotated in the 26695 genome). A pair of oligonucleotide primers was used to detect the presence of the *cagA* promoter region and the LEC (left-end region of *cag* PAI) containing both inside and outside genes of *cag* PAI (Ikenoue et al., 2001). The promoter region of the *cagA* gene was found in all the *cag*-positive strains (Fig. 1). With internal primers (LECF1-LECR1) for the left-end region, only 19 of 48 *cag*-positive (39.6%) strains were amplified. With primer pair LECF2-LECR2, 95.8% *cagA*-positive strains (46/48) gave a positive amplicon.

CagA expression and translocation

Then we addressed the question whether the *cagA* gene of *H. pylori* strains isolated from DU and NUD/AV can uniformly express the mRNA and the protein. First, we performed the RT-PCR analysis of the *cagA* gene of representative strains from both groups. All the tested strains isolated from both DU and NUD/AV subjects expressed the *cagA* transcripts at the transcriptional level indicating the intact *cagA* promoter regions of these strains (Fig. 8). We then went on to perform the CagA expression by Western blot analysis after infecting the AGS cells for 5 h with *H. pylori* strains isolated from both groups (Fig. 9). Expression of CagA indicated that the type IV secretion system (T4SS) is functional in the NUD/AV group also as CagA is translocated from bacteria to the host cell through this machinery.

Correlation with clinical outcome

The frequency of complete *cag* PAI, partially-deleted *cag* PAI, and deleted *cag* PAI among the duodenal ulcer patients and NUD or AV were 80% (24/30) and 86.4% (19/22), 6.6% (2/30) and 13.7% (3/22), and 13% (4/30) and 0%, respectively (Table 3). These differences were, however, not significant ($p>0.05$). All the 48 *cagA*-positive strains isolated from DU patients and asymptomatic individuals carried the gene *cagE*. *cagT* gene was present in 100% of the DU patients and in 86.4% (19/22) of the strains from NUD or AV. All the 4 *cag* PAI-negative strains belonged to the DU group.

Discussion

The severity of *H. pylori*-related disease correlates with the presence of a *cag* pathogenicity island (*cag* PAI) in western countries. Genetic diversity within the *cag* PAI may have a profound effect on the pathogenic potential of the infecting strain, and the *cag* PAI has been studied in different *H. pylori* populations by various methods including PCR, Southern

blotting, dot blot, and by long-distance PCR (Slater et al., 1999; Audibert et al., 2001; Ikenoue et al., 2001). In this study, we comprehensively analyzed 52 *H. pylori* strains isolated from DU and NUD/AV from Kolkata, India, by PCR using 83 primers sets. Our results showed that 92.3% of the strains were positive for *cagA*. These strains were also positive for the promoter region of *cagA*. Strains that were *cagA*-negative (7.7%) were also negative for all the genes of *cag* PAI. We found a partially deleted *cag* PAI in only 9.6% of the strains, while a complete *cag* PAI was present in the 82.6% of the strains. These data are consistent with our previous observation (Mukhopadhyay et al., 2000) and are different from the reports from East Asia (Japan and China) (Ito et al., 1997; Pan et al., 1997) and western countries (Nilsson et al., 2003). An analysis from East Asia (Ito et al., 1997; Pan et al., 1997) and South Africa (Kidd et al., 2001) revealed that 100% of the *H. pylori* strains are cag^+. Studies in Swedish (Nilsson et al., 2003) and French populations (Jenks et al., 1998) indicated the frequency of the *cagA* gene in 83% and 87.7%, respectively. Our results on partially deleted *cag* PAI are similar to the observations from the East Asia (Ikenoue et al., 2001) and also from the Western world (Censini et al., 1996; Jenks et al., 1998; Maeda et al., 1999; Nilsson et al., 2003). Indian populations belong to a diverse set of culture and language groups that are largely endogamous. As a result of evolutionary antiquity and endogamy, populations of India show a high genetic differentiation and extensive structuring (Majumder, 2010). The geographical distribution of the language groups in India is largely non-overlapping. Linguistic differences of populations provide the best explanation of genetic differences observed in this region of the world (Majumder, 2010). Interestingly, a recent report from the southern part of India (Ali et al., 2005) showed the presence of intact *cag* PAI in 39.7% and partially deleted *cag* PAI in 58% of the strains. These strains were collected from the population of South India of the Telugu linguistic group who are mainly Dravidian and married consanguineously for millennia and are different from other Indian populations (Ahmed et al., 2003). The genetic separation of this population from other Indian communities has already been reported (Bamshed et al., 1996). A low prevalence of intact *cag* PAI was also reported from Karachi, Pakistan, with a missing *cagE* and *cagA* promoter region (Yakoob et al., 2009).

Therefore, our results showed the diversity of the prevalence of *cag* PAI among the different regions of India. Another study (Kauser et al., 2004) reported the analysis of *cag* PAI of *H. pylori* strains from various regions of the world including Japan, India, England, and Latin American countries using the same primer set with the prevalence of intact *cag* PAI in 57.1%, 12%, 3%, and 18.6% of the cases, respectively. This observation is markedly different from other reports on intact *cag* PAI positivity from the same regions (Jenks et al., 1998; Ikenoue et al., 2001). Some studies might have used smaller numbers of primer sets to analyze the *cag* PAI region, and this may lead to a lower percentage of intact *cag* PAI. Moreover, the same primer sets may not work in different regions as region-specific variations in the *cag* PAI have been reported from different parts of the world. In fact, 29% of the *cag* PAI genes of Kolkata strains were also undetected in our initial PCR assay using primer set A. However, our subsequent PCR analyses using primer set B and primer set C (Table 2) increased the detection level to 95%.

The relationship between the presences of complete, partially deleted, and absent *cag* PAI with duodenal ulceration is controversial. In our study, *cag* PAI was present in 86.6% and 100% of DU and NUD/AV, respectively. The complete *cag* PAI and partially deleted *cag* PAI were present in 80% and 6.7% of DU and 86.4% and 13.6% of NUD/AV, respectively, while 13.9% of DU and 0% of NUD/AV were cag^-. These results are in accord with our previous reports, which showed a high prevalence of cag^+ strains in both peptic ulcer patients and benign-disease populations (Mukhopadhyay et al., 2000). Many studies in European and Asian populations (Jenks et al., 1998; Maeda et al., 1999; Kidd et al., 2001; Nilsson et al., 2003) have reported the presence of increased intact *cag* PAI in duodenal

ulcer patients compared to gastritis controls. However, several reports indicated a lack of correlation between the presence of *cag* PAI and peptic ulcer disease (Peters et al., 2001; Zhang et al., 2005). Moreover, *cag* PAI-negative strains, like the present study, have been detected among duodenal ulcer patients (Nilsson et al., 2003). Interestingly, in the present study, all the cag^- strains were isolated from DU patients. This may also be due to the fact that the cag^- strains that we have isolated do not represent the entire *H. pylori* population of the whole stomach since different *H. pylori* strains may have colonized different parts of the stomach. It is possible that cag^+ *H. pylori* strains were also present in a different area of the stomach of the same DU patient. Moreover, our unpublished data strongly suggest that the coexistence of multiple *H. pylori* strains in a single host, unlike the situation in Europe and North America (Taylor et al., 1995; Marshall et al., 1996; Shortridge et al., 1997), are quite common in India. Moreover, Yakoob et al. (2000) showed that 2 different *H. pylori* strains may coexist in an infected individual and may not be uniformly distributed among biopsy sites. It has been shown that if both forward and reverse primers are selected from same ORF, it produces better results than taking 2 primers from 2 different ORFs especially in cases of detection of false-negative PCR. Therefore, we used pairs of oligonucleotide primers to detect the presence of the *cagA* promoter region and the LEC containing both inside and outside genes of *cag* PAI (Ikenoue et al., 2001). In our study, LEC1 was found to be present in only 19 of the 48 (39.6%) *cag*-positive strains amplified, and LEC2 was present in 46 of the 48 *cag*-positive strains (95.8%). These observations are similar to those of other studies (Ikenoue et al., 2001).

The *cagY* gene encoding a protein, homologue of virB10, is relatively large (5–6 kb) and includes 2 repetitive regions (Liu et al., 1999), and an amplified fragment produced by PAI14S and PAI15AS primers contained the first repetitive region, which exists in the 5’ end of the gene (from the 9th AA in 26695). In our study, for *cagY* gene, 66.7% of the strains gave a shorter amplicon than 26695. We also observed that the repetitive region consists of 2 complete units (390 bp each) and one incomplete unit (171 bp) or one complete and one incomplete unit. Further, analysis showed that the strains having shorter amplicons lacked one complete unit (390 bp) and therefore had a 390-bp deletion, although this deletion did not affect the reading frame and mRNA expression. Moreover, CagA expression of *H. pylori*-infected AGS cells by Western blot analysis indicated that T4SS is uniformly functional in DU and NUD/AV groups (even when HP0527 is shorter) as CagA is translocated from bacteria to the host cell through this machinery. The HP0535 gene was found to be highly diverse among the Kolkata strains. We found that insertion occurs within the HP0535-HP0536 gene. Among 46 strains that amplified, 31 (67.4%) gave a higher amplicon with respect to 26695 when PCR was done for HP0535 and HP0536 gene.

In conclusion, it was found that 82.6% of the *H. pylori* strains of Kolkata had a complete *cag* PAI, but 92.3% of the strains carried the *cagA* gene. Thus, the presence of the *cagA* gene does not necessarily indicate the presence of intact *cag* PAI and cannot be used invariably as a marker of intact *cag* PAI or as a marker for virulence, especially in the context of Indian *H. pylori* strains. Although the *cag* PAI is an important virulence component for infection, the determination of the direction of disease development (gastritis, ulcers, or cancer) is likely to involve a highly complex interplay of many bacterial and/or host factors. The differences in the development of *H. pylori*-induced diseases could also be due to the fact that the strains, upon colonization, may modify their abilities to induce epithelial cell responses (e.g. IL-8 secretion) as part of their adaptation to the changing conditions within the host milieu (Backert et al., 2004). In order to unravel the hidden features of *H. pylori*–host interactions, our results suggest that future work should be directed toward the identification of the host genetic background, other translocated bacterial virulence factors, and probable environmental factors, which may play a crucial role in the formation of *H. pylori*-related gastroduodenal disorders.

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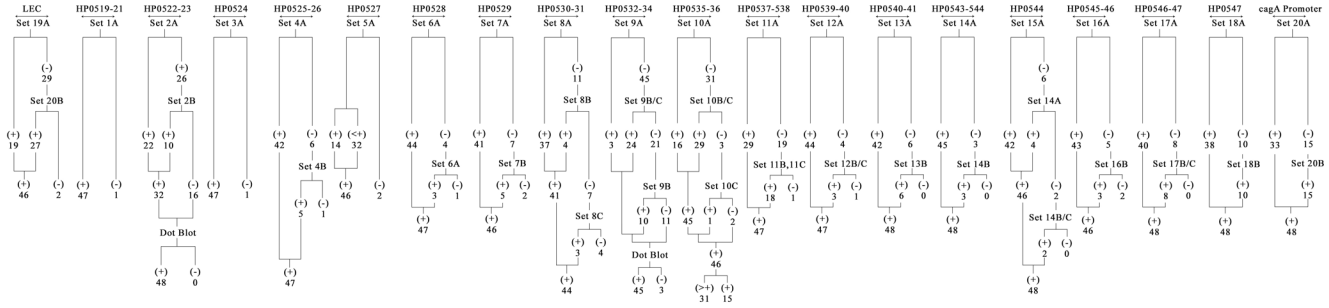


Fig. 1.

Schematic representation of the distribution of the genes in *cag* PAI by PCR using 83 primers in various combinations and dot-blot hybridization among the *H. pylori* strains in Kolkata. Here, '+' means positive by PCR and amplicon size is the same as in 26695, whereas '>+' indicates positive but amplicon size is more than that of 26695 which is used as a reference positive control.

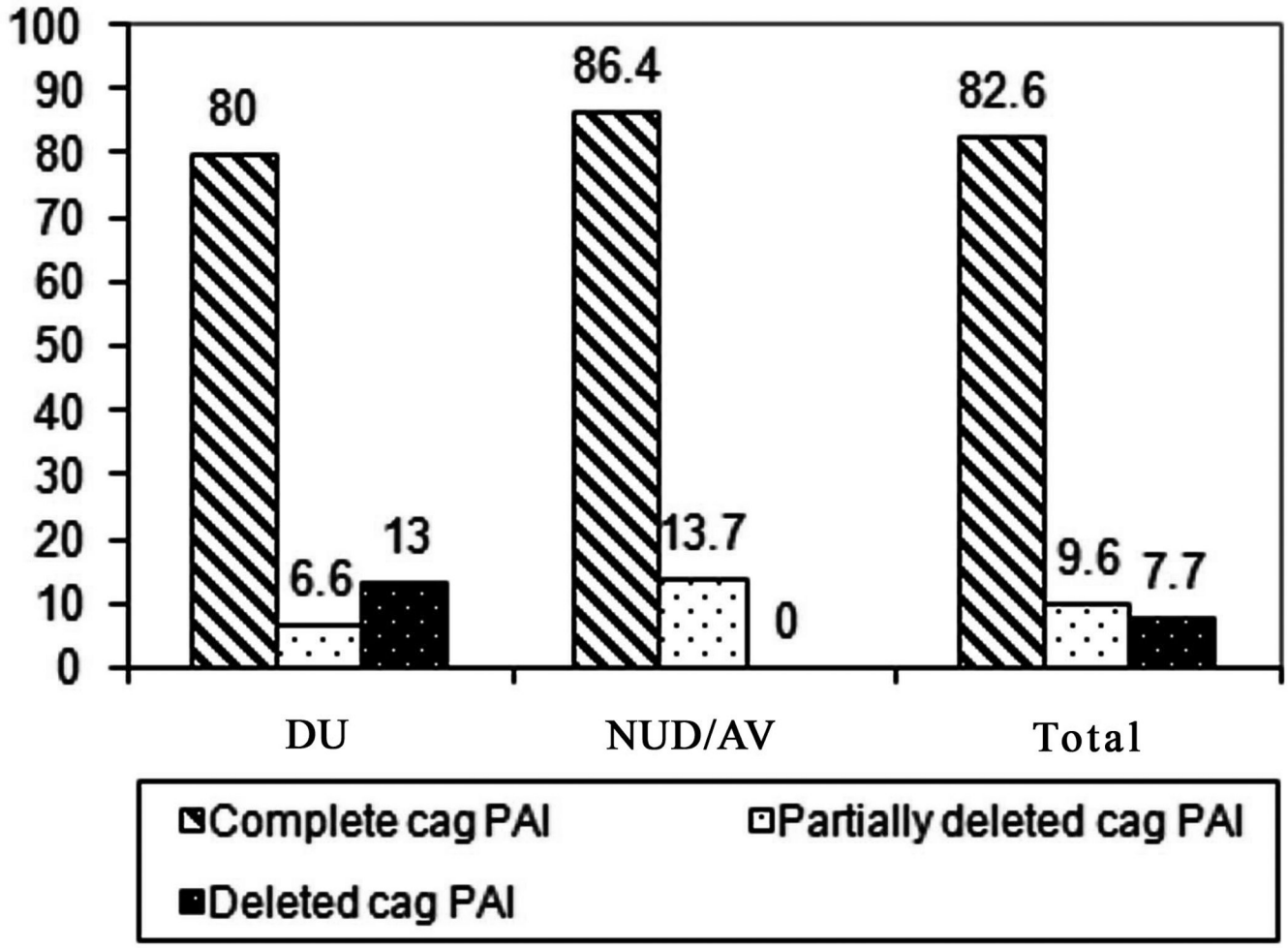


Fig. 2.

Percentage of intact *cag* PAI and partially deleted *cag* PAI and deleted *cag* PAI among the Kolkata strains with different clinical outcome. In total, 82.6% of the strains have intact *cag* PAI while only 9.6% of the strains have partially deleted *cag* PAI. Only 7.7% have *cag* PAI with a complete deletion. The frequency of complete *cag* PAI, partially deleted *cag* PAI, and deleted *cag* PAI among the duodenal ulcer patients and asymptomatic volunteers/non-ulcer dyspepsia were 80% and 86.4%, 6.6% and 13.7%, and 13% and 0%, respectively.

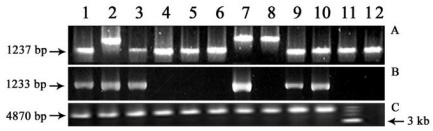


Fig. 3.

(A) PCR assay of representative strains from Kolkata using primer set PAI-1S and PAI-1AS amplifying part of HP0519, HP0520, and HP0521. Lane 1 represents 26695 while lanes 2–12 represent different clinical isolates from Kolkata.6

(B) PCR assay of the same set of strains as in (3A) using primer set PAI-3S-PAI-3AS amplifying part HP0522 and HP0523.

(C) PCR assay of representative strains using primers PAI-1S and PAI-6AS designed from HP0519 and HP0524 gene of 26695, respectively. Lane 1 represents 26695, lanes 2–10 represent the strains that were negative for HP0522-523 by PCR, lane 11 is a 1-kb marker, and lane 12 indicates a *cag* PAI-negative strain as a negative control.

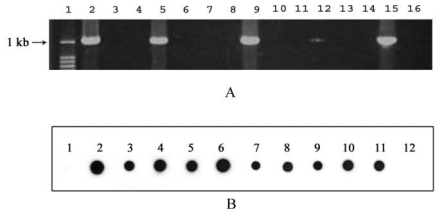
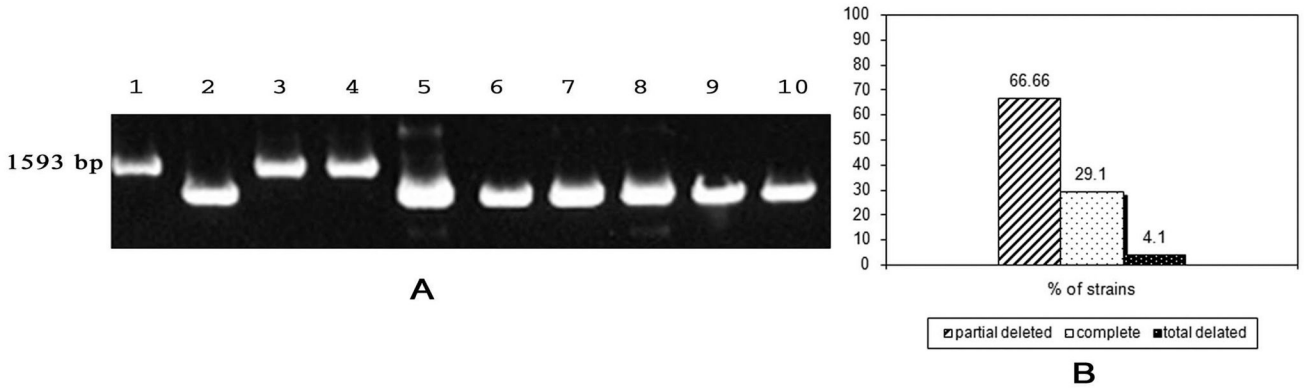
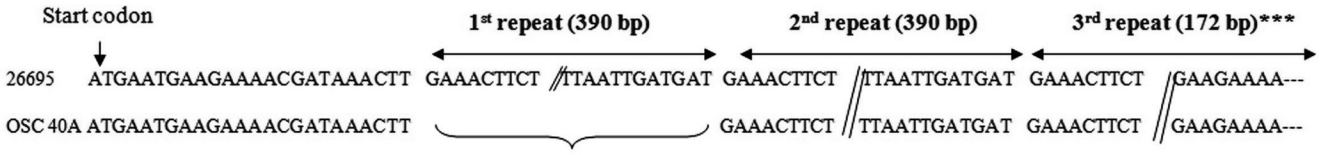


Fig. 4.

(A) Representative PCR result of HP0532–HP0534 gene using primer set PAInew2F-PAI25AS among the Kolkata strains. Lane 1, 100-bp marker (New England Biolab); lane 2, positive control; lane 5, 9, and 15 gave positive amplicons; lane 16, negative control. PCR assay yielded amplicons in 3 strains among 48 *cag* PAI-positive strains tested with the primer pair PAInew2F-PAI25AS.

(B) Dot-blot hydbridization result of representative strains showed positive signals of the PCR-negative strains using the PAInew2F-PAI25AS-amplified 1-kb fragment from 26695 as probe. '1' in figure denotes AM1, *cag* PAI-negative strain (as a negative control), and '2' is 26695 (as a positive control), and '3–12' denotes the same strains as in lanes 2–11 of Fig. 4A.





*** The 3rd repeat is not complete and only a portion of the 1st two repeats are present

C

Fig. 5.

(A) PCR assay of the representative strains of *H. pylori* from Kolkata with primers PAI-14S and PAI15AS amplifying a part of HP0527 (VirB10) showed shorter amplicon in most of the strains as compared to 26695. Lane 1 represents 26695 while lanes 2–10 represent different strains isolated from Kolkata. (B) Percentage of *H. pylori* strains having partially deleted, complete, and totally deleted HP0527 gene of *cag* PAI among Kolkata strain. 66.7% of the strains gave a shorter amplicon, 29.1% of the strains gave the same amplicon as that of 26695 while only 4.1% of the strains showed complete deletion of the gene.
(C) Nucleotide sequence alignment of 26695 and one representative strain having shorter amplicon of the HP0527 gene of *cag* PAI from Kolkata. The first repeat of 390 bp is absent in those strains that have a shorter gene length in the HP0527 gene.

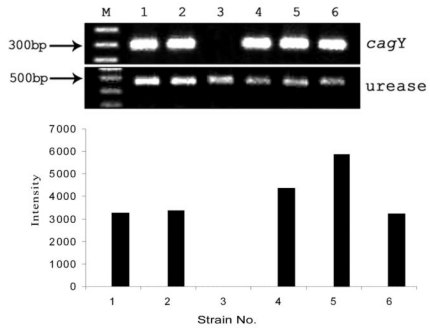


Fig. 6.

RT-PCR analysis of mRNA levels for the detection of the *cagY* gene using primers HPO527F and HPO527R and urease gene using primers UreBF and UreBR. M, 100-bp marker strains. Lane 1, 26695; lane 2, I-34; lane 3, S(New England Biolab). Lanes 1–6, signifies amplification *cagY* and urease gene of *H. pylori* an61; lane 4, San74; lane 5, San10; lane 6, San54. Lanes 1and 4 denote the presence of 2 complete units (390 bp each) and one incomplete unit; lanes 2, 5, and 6 denote the presence of one complete and one incomplete unit. Results under the panels A and B estimate the amount of *cagY* gene transcripts by semiquantitative RT-PCR. Data for *cagY* mRNA were normalized to data for urease. The results were analyzed with Quantity One software (Bio-Rad).

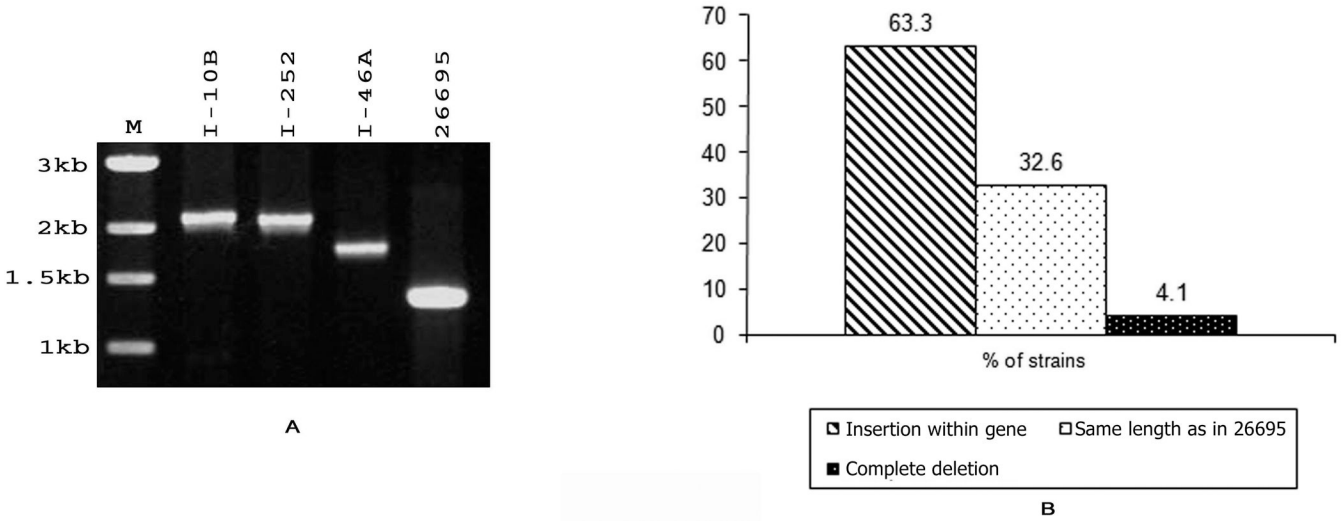


Fig. 7.

(A) PCR amplification of representative *H. pylori* strains from Kolkata showed variations in the gene length of HP0535-HP0536 using primer set PAI27S-PAI27AS. Lane 1, 1-kb marker (New England Biolab); lanes 2–5, I-110, I-252, I-46A, and 26695. I-252 and I-110 gave a 2-kb amplicon while I-46A gave about 1.8-kb amplicon. Among 47 strains that amplified with the primer pair, 31 gave a higher amplicon than that of 26695.

(B) Percentage of *H. pylori* strains showing variation in the gene length of HP0535–HP0536 gene using primer PAI27S-PAI27AS isolated from patients in Kolkata. About 63.3% of the Kolkata strains have an insertion within the gene.

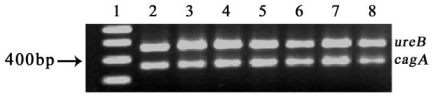


Fig. 8.

RT-PCR analysis of mRNA levels for the detection of the *cagA* gene of *H. pylori* strains isolated from NUD/AV (lanes 2, 6, 7, and 8) and DU (lanes 3–5) using primers Cag 5cf and Cag 3cr and *ureB* using primers UreBF and UreBR. Lane 1 is a 100-bp marker.

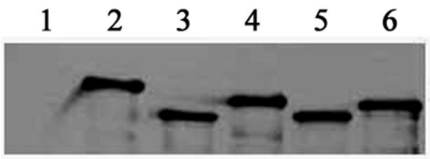


Fig. 9.

AGS cells were cocultured with *H. pylori* strains from DU (lanes 3 and 4) and NUD/AV (lanes 2, 5, and 6) subjects for 5 h at 37°C before the cells were lysed and the samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting with polyclonal anti-CagA antibody. Lane 1 indicates the result of AM1 (one *cag* PAI-negative strain) infected AGS cell.

Table 1

Sequences and locations of the oligonucleotide primer sets used in this study.

(a) List of *Helicobacter pylori cag* pathogenicity island (PAI)-spanning primer sets designed from the strain 26695.					
Primer position in 26695	**Designation (Tomb et al., 1997)**	**PAI primer**	**Nucleotide sequence (5'-3')**	**Primer set**	**Size of PCR product (bp)**
546899	HP0519	PAI-1S	ACACTGCCAAGCCCGATGCTGTA		
548162		PAI-1AS	GATACAGCGGTTGCTAGT	PAI-1S- PAI-1AS	1264
547464	HP0520	PAI-2S	GTGTCTTGAGCGGTGCTATG		
548829		PAI-2AS	ATCTCTTAGGGGCGAACACACTTC	PAI-2S- PAI-2AS	1366
547652		HP0520AS1	CAGTTGGTTCGTTGGTAAC		
547850		HP0521S1	GCTGTAAGGGCGTTTTAC		
549312		HP0522AS3	GATCAAAGTCCCCTCATAGC		
548547		PAI-3S	CATCACAGGCTCATTAGAG		
549779		PAI-3AS	CTGTTGTTCACCCTAGAGAG	PAI-3S- PAI-3AS	1233
549703	HP0523	PAI-4S	GGTTGCGACAATGAAGTG		
550007		PAI-5S	GCGCTTACAATGGGGAATGAA		
550666		PAI-6S	TCTTAGCGCCATTCCTACCATAACC		
551769		PAI-6AS	CGTTCATTGGCTTGATTGCTCCTAC	PAI-6S- PAI-6AS	1104
552610	HP0525	PAI-8S	GGCCAAACGGATAAACGCTTCTTCA		
553630	HP0526	PAI-8AS	GTGGCGTTTCAGATCCTAGGGATAG	PAI-8S- PAI-8AS	1021
553964		PAI-new 1R	GCCTCTTCTTTAAAAGATAGCAAC		
558446		PAI-14S	CAATCTAGCGCCACTTGAAC		
558925		PAI-15S	CACGATAAGAACAGCGACTAC		
560058		PAI-15AS	CTATGGTGAATTGGAGCGTGTG	PAI-15S-PAI-15AS	1134
560207	HP0528	PAI-17S	CAATGGCGGCATCAGTCATGCTCAA		
561183		PAI-17AS	ACTTATCGTAGATGCGCCTGACC	PAI-17S-PAI-17AS	977
561843	HP0529	PAI-20S	TAGCAACAGAGGGCGTTATG		
562568		PAI-19AS	TCAAAGGAGCGGACGCTGCTGTT		
562972		PAI-20AS	CACAAGTTTAGCCGCTAGCA	PAI-20S-PAI-20AS	1130

(a) List of *Helicobacter pylori cag* pathogenicity island (PAI)-spanning primer sets designed from the strain 26695.					
Primer position in 26695	**Designation (Tomb et al., 1997)**	**PAI primer**	**Nucleotide sequence (5'-3')**	**Primer set**	**Size of PCR product (bp)**
563540	HP0530	PAI-22S	GATAGCTTCTGCTCGGACTT		
564932		PAI-new 2F	CAAAGAAACAAGGCGGTGCA		
565031		PAI-23AS	TGCACCGCCTTGTTTCTTTG		
564889		PAI-24S	GTAGCACTAACGACAAGGTGCT		
565975		PAI-24AS	TAACGCCCGTTGGCGTTTCTCT	PAI-24S-PAI-24AS	1087
565573		PAI-25S	CTTGCATGGCTATGATGTGAG		
566555		PAI-25AS	GCATACAAACAAGGGAGCGTTAG	PAI-25S-PAI-25AS	983
567109		PAI-26AS	TCATCTTTCACGCAGAGC		
566816		PAI-27S	CAGAGCGGTCATAATTCAAAGAGC		
567212		HP0535S2	CCAACCAAAGCAGATCCCATGT		
568157	HP0536	PAI-27AS	CTTATGGGGCAGGGGTGATTTTAG	PAI-27S-PAI-27AS	1342
567426		HP0535AS2	TTGTTGGGTGGCGGAACAAA		
567409		PAI-28S	TGTTCCGCCACCCAACAAAGAA		
568993	HP0537	PAI-30S	GAGGCTCTAGAGAAAGAGAC		
569303		PAI-29 AS	GCTAATCGGCTCGCTTTT		
569724	HP0538	PAI-31 S	CGTAGATAGCGATCCTATG		
570078	HP0538	PAI-30AS	CTCTCAAAGCGTTAGTGG	PAI-30S-PAI-30AS	1086
570169		PAI-32S	AAGCGGCTAAGCACAAAG		
571054	HP0539	PAI31AS	CACAGACGCTTGTAGAAAG		
571040		PAI33S	CTACAAGCGTCTGTGAAG		
571479	HP0540	PAI-32AS	AGAGACCAACCAACAAGTGC	PAI-32S-PAI-32AS	1311
571729		PAI-34S	GCGCGTTCAAATCTACTG		
572648	HP0541	HP0540AS3	GCTTGAACCCGCCTTAAA		
573078		PAI-34AS	CACTCCTGCATGCCCTATTG	PAI-34S-PAI-34AS	1350
574444	HP0543	PAI-37S	GCTTCAACGCTCATATCAG		
574614		PAI-36AS	CATAAGCGAGGACATGCAGAAC		
574991	HP0544	PAI-38S	GAGCGGTAAGGTTTTGTTCGGTGAT		

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(a) List of *Helicobacter pylori cag* pathogenicity island (PAI)-spanning primer sets designed from the strain 26695.					
Primer position in 26695	**Designation (Tomb et al., 1997)**	**PAI primer**	**Nucleotide sequence (5'-3')**	**Primer set**	**Size of PCR product (bp)**
575532		PAI-37AS	GTCAGACTTGCGACTCAAAG	PAI-37S-PAI-37AS	1089
576094		PAI-39S	GCCGCCCAAGCAAAAGGATTTA		
576584		PAI-38AS	CCAACGCAGCGACTTTCTCTATG		
577589		PAI-39AS	ATGGGGTGATCCTTACTAACAACTA	PAI-39S-PAI-39AS	1498
578305		PAI-40AS	CCAACAAACAACGCTGCTTTC		
578096	HP0545	PAI-41S	GCCACAAACACCCCTCTCTTTA		
579016		PAI-41AS	CAATCCTTTAATGGCGGTCACCAG	PAI-41S-PAI-41AS	921
578847	HP0546	PAI-42S	CAGTCGCCTGACCTCTTTTGAT		
580440		PAI-42AS	CTGAAAAGATTGTTTGGCAGA	PAI-42S-PAI-42AS	1594
579910	HP0547	PAI-43S	AAGGAGAAACAATGACTAACGAAACTATTG		
581979		PAI-45S	GAATTGTCTGATAAACTTGAAA		
583130		PAI-45AS	GCGTATGTGGCTGTTAGTAGCG	PAI-45S-PAI-45AS	1152
584021		PAI-46AS	GTTGATGCTCCCCTTCAACA		
579530		HP0546AS1	TTTGGTTTGTGTGTGTCATACT		

(b) List of *Helicobacter pylori cag* pathogenicity island (PAI)-spanning oligonucleotide primer sets designed from the accession numbers U60176 and AC000108.					
Gene of DNA region amplified	**Primer**	**Primer sequence**	**Primer set**	**Amplicon size (bp)**	**Reference**
LEC	LecF1	ACATTTTGGCTAAATAAACGCTG			Ikenoue et al. (2001)
	LecR1	TCTCCATGTTGCCATTATGCT	LecF1- LecR1	384	Ikenoue et al. (2001)
	LecF2	ATAGCGTTTTGTGCATAGAA			Ikenoue et al. (2001)
	LecR2	ATCTTTAGTCTCTTTAGCTT	LecF2- LecR2	877	Ikenoue et al. (2001)
cagE	cagE-F1	GCGATTGTTATTGTGCTTGTAG		329	Ikenoue et al. (2001)
	cagE-R1	GAAGTGGTTAAAAAATCAATGCCCC	cagE-F1- cagE-R1		Ikenoue et al. (2001)
cagT	cagT-F1	CCATGTTTATACGCCTGTGT		301	Ikenoue et al. (2001)
	cagT-R1	CATCACCACACCCTTTTGAT	cagT-F1- cagT-R1		Ikenoue et al. (2001)
cagA promoter	cagAP-F1	GTGGGTAAAAATGTGAATCG	cagAP-F1-PAI-42AS	730	Ikenoue et al. (2001)

(b) List of *Helicobacter pylori cag* pathogenicity island (PAI)-spanning oligonucleotide primer sets designed from the accession numbers U60176 and AC000108.					
Gene of DNA region amplified	**Primer**	**Primer sequence**	**Primer set**	**Amplicon size (bp)**	**Reference**
	cagAP-F2	CTACTTGTCCCAACCATTTT	cagAP-F2-PAI-42AS	1181	Ikenoue et al. (2001)
cagA	cag 5cf	GTTGATAACGCTGTCGCTTCA			Chattopadhyay et al. (2003)
	cag 3cr	GGGTTGTATGATATTTTCCATAA	Cag 5cf-cag 3cr	350	
cag PAI empty site	Lunil 1	ACATTTTGGCTAAATAAACGCTG			Mukhopadhyay et al. (2000)
	R5280	CCAACGTGCGTAAAAGGGAATTAG	Lunil 1-R5280	550	

Table 2

Percentage of detection of various *cag* PAI genes using the different primer sets A, B, and C.

		Primer set							
No.	**Gene**	**Primer set A (n=48)**	**Detection (%)**	**Primer set B**	**Detection (%)**	**Primer set C**	**Detection (%)**	**(B+C)**	**(A+B+C)**
1	HP0519, 520, 521	PAI-1S- PAI-1AS	47/48 (95.7%)						47/48 (95.7%)
2	HP0522, 523	PAI-3S- PAI-3AS	22/48 (45.7%)	PAI-3S- PAI-6AS	10/26 (38.5%)	*a*		38.5%	32/48(66.6%)
3	HP0524	PAI-6S- PAI-6AS	47/48 (97.8%)					47/48 (97.8%)	
4	HP0525-526	PAI-8S & PAI new1R	44/48(87.36%)	PAI8S-PAI8AS	5/6 (83.3%)			83.3%	47/48 (97.7%)
5	HP0527	PAI14S-PAI15AS	46/48 (95.7%)						46/48 (95.7%)
6	HP0528	PAI-17S & PAI 17AS	44/48 (91.5%)	PAI 16S - PAI 17AS	3/4 (75%)			75%	47/48 (97.7%)
7	HP0529	PAI-20S & PAI 20AS	41/48 (85.2%)	PAI 20S-PAI 19AS	5/7 (71.4%)			71.4%	47/48 (97.7%)
8	HP0530-531	PAI22S- PAI 23AS	37/48 (76.9%)	PAI 22S- PAI 25AS	4/11 (36.4%)	PAI22SHP0535AS2	3/7 (42.9%)	63.6%	44/48 (91%)
9	HP0532-534	PAInew2F- PAI 25AS	3/48 (6.2%)	PAI 24S- PAI 25AS	24/45 (53.3%)	PAI 24S- 26AS	10/21(47.6%)	75.5%	37/48 (76.7%)
10	HP0535-536	PAI27S - PAI 27AS	16/48 (33.3%)	PAI27S-HP0535AS2	29/31 (93.5%)	HP0535S2-PAI27AS	1/3 (33.3%)	96.7%	46/48 (95.6%)
11	HP0538-538	PAI30S - PAI 30AS	29/48 (60.3%)	PAI31S - PAI 30AS	18/19 (94.7%)	PAI30S - PAI 29AS		94.7%	47/48 (97.7%)
12	HP0538-539	PAI32S - PAI 32AS	44/48 (91.5%)	PAI 31S-PAI 32AS	3/4 (75%)	PAI32S-PAI31AS		75%	47/48 (97.7%)
13	HP0540-541	PAI34S - PAI 34AS	42/48 (87.4%)	PAI34S-HP0540AS3	6/6 (100%)			100%	48/48 (100%)
14	HP0543-544	PAI37S - PAI 37AS	45/48 (93.6%)	PAI37S - PAI 36AS	3/3(100%)	PAI38S-PAI37AS		100%	48/48 (100%)
15	HP0544	PAI-39S- PAI 39AS	42/48 (87.36%)	PAI37S - PAI 36AS	2/4 (50%)	PAI38S-PAI37AS	2/2 (100%)	100%	48/48 (100%)
16	HP0545-546	PAI41S - PAI 41AS	43/48 (89.4%)	PAI 41S-PAI 40AS	3/5 (60%)			60%	46/48(95.7%)
17	HP0546-547	PAI42S- PAI 42AS	40/48 (83.2%)	PAI42S- PAI-41AS	8/8 (100%)	PAI43S-PAI42AS		100%	48/48 (100%)
18	HP0547	PAI-45S -45AS	38/48 (79%)	cag5cf-cag3cr	18/18 (100%)			100%	48/48 (100%)
19	Left-end region of *cag* PAI	LecF1-LecR1	19/48 (39.5%)	LecF2-LecR2	27/29 (93.1%)			93.1%	46/48 (95.7%)
20	Promoter region of *cag* PAI	cagAPF1-PAI42AS	33/48 (68.6%)	cagAPF2-PAI42AS	15/15 (100%)			100%	48/48 (100%)
Mean			**71%**		**77.9%**		**56%**	**84%**	**95%**

[a]PCR assay by PAI-1S and PAI-6AS (designed from HP0519 and HP0524, respectively) yielded around 4.8-kb amplicon and indirectly indicated that the remaining 16 strains also contained HP0522-523 (Fig. 3C). As these primers do not fall into the HP0522-23 region, it was not included in the above table.

Table 3

Distribution pattern of intact *cag* PAI and partially deleted *cag* PAI/*cag* PAI^{-} in *H. pylori* strains isolated from DU and NUD/AV subjects in Kolkata, India.

	DU	NUD/AV	Total
Intact *cag* PAI	24	19	43
Partially deleted *cag* PAI/*cag* PAI^{-}	6[a]	3[b]	9
Total	30	22	52

[a] Among 6 strains, 4 strains produced total *cag* PAI^{-}, and the remaining 2 amplified partially deleted *cag* PAI.

[b] All 3 strains yielded partially deleted *cag* PAI.