# 8. Stduies on *Helicobacter pylori*

## 8.1 Characterization of *cag* pathogenicity island (*cag* PAI) of *Helicobacter pylori* strains from patients in Kolkata

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*Helicobacter pylori* is of growing concern today because of its crucial role in the pathogenesis of chronic gastritis, peptic ulcer diseases and in the multi-step carcinogenic process of gastric cancer. Although the pathogen is present in almost in all patients, but it is still an enigma why only a small proportion develop symptomatic disease. The CagA protein of *H. pylori* is an immunogenic antigen, which seems to be associated in eliciting the disease process. Based on the presence or absence of *cag* PAI, *H. pylori* strains can be divided into two types. Type I strain carry the *cag* PAI, a 37 kb sequence consisting of 27 putative genes and is often associated with a severe disease outcome of *H. pylori* infection in patients. The *cagA* gene (encoding the cytotoxin associated antigen A) is a marker for this locus, which is absent in type II strains. In the *H. pylori* infection model of Mongolian gerbils, Ogura *et al*. (2000) demonstrated recently that type I *H. pylori* strains with an intact *cag* pathogenicity island induced strong inflammation and ulceration in the stomach, whereas an isogenic mutant with a defect in the type IV secretion system was unable to elicit such a response. It has been recently shown that the genes of the *cag* PAI actually encode a functional type IV secretion system represented by the *Agrobacterium tumefaciens* virB operon, and that *H. pylori* delivers the CagA protein into the cultured gastric epithelial cells. Seventeen out of 27 genes were found to be absolutely essential for the translocation of *cagA* into the host cells and 14 out of 27 for the ability of *H. pylori* fully to induce transcription of IL-8.

**Analysis of the *cag* PAI -** In this study we analyzed the composition of the *cag* PAI in 52 clinical isolates from West Bengal population with 79 primers with various combinations. Among the 52 clinical isolates 30 were from Duodenal ulcers and 22 were from Healthy volunteers. PCR was performed on all isolates with primers detecting the *cag* PAI genes and the primers complementary to the flanking regions of the *cag* PAI, which generated a fragment when PAI was absent. Only those strains which failed to amplify with the standard primer set for a gene were checked further by PCR using primers within and outside the gene designated as either 2$^{nd}$ set of primer or 3$^{rd}$ set of primer or both. Out of 52 strains, only four strains (7.7%) were *cag* PAI negative. In the *cag* PAI empty site PCR where primers were designed from the flanking region of the *cag* PAI, these four strains gave an amplicon of 550 bp indicating a complete lack of the *cag* PAI.

**Dot Blot Analysis -** After initial screening by PCR, it was found that some sets of primers failed to provide any amplicon in most of the Kolkata strains analyzed in this study. To understand whether these ORFs were truly absent from these strains, they were subjected to dot blot hybridization study. The specific primers, which failed to produce amplicon from the Kolkata strains, were used to amplify the gene from 26695 and this amplified segment was used as probe in Dot Blot analysis. In this study we found that all strains negative by PCR for a segment of a gene, hybridized with the probe in Dot Blot. For example, 16 strains were negative by PCR for HP0522-523 gene, but showed positive signals after hybridization with the specific probe generated by the same primers from 26695. Similarly 45 strains were negative by PCR with primer set for HP0532-HP0534 gene (Fig 8.1.1a). However, after dot blot hybridization with the specific probe generated by the same primer set from 26695, 45

out of 48 *cag* PAI positive strains gave positive signals (Fig 8.1.1b). This failure of the primers to amplify the gene fragment by PCR may have been due to some mismatch or any deletion at the primer binding site. So this result provided us an impetus to design new sets of primers both from inside and outside of the gene of interest and to check the presence or absence of each gene by two or more sets of primers.

**HP0527 gene of *H. pylori* -** HP0527 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, specialized complex that transfer a variety of multimolecular complexes across the bacterial membrane to the extracellular space or into other cells. Recently, a model was proposed, where HP0527 serves as a variable antigenic protein located on a novel filamentous surface organelle. To understand the distribution of the gene encoding this HP0527 protein among the *cag* PAI positive strains, two primers were designed from HP0527 and HP0528 genes of 26695. PCR assay using these two 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 amplicon than the standard 26695 strain while 14 of 48 strains (29.1%) gave an amplicon same as that of 26695 (Fig 8.1.2). To understand more closely, we performed the sequence analysis of the few representative shorter amplicons. Careful analysis of the sequences revealed that the strains having a shorter amplicon had a 390 bp deletion, although this deletion did not affect the translation of the protein.

**HP0535 Gene of *cag* PAI -** With the primer pair designed from HP0535 and HP0536 gene of 26695, only 17 out of 48 *cag* PAI positive strains gave a positive amplicon (Fig 8.1.3). Negative result by PCR may be due to either an absence of tested gene or a lack of primer annealing due to interstrain variation in the sequence targeted by the primer. Therefore all the strains, which did not amplify, were checked with two more internal primers. These two primer set were able to amplify all the strains excepting two. However, among 46 strains that amplified, 31 of them gave a higher amplicon (Fig 8.1.4).

**The *cagA* gene and *cagA* promoter region and LEC of the *cag* PAI -** Primers cag5cf and cag3cr were designed from conserved regions to amplify 350 bp (positions 109 to 459 of *cagA* of strain 26695). All the 48 *cag* PAI strains gave a 350 bp amplicon with cag5cf and cag3cr primers. All the *cag* 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), coding for the predicted ribose 5-phosphate isomerase and a membrane protein. Pairs of oligonucleotide primers were 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 and these primer pairs were taken from Ikenoue *et al.*, 2001. With internal primers LECF1-LECR1 only 19 of 48 *cag* positive (39.6%) strains were amplified. With primer pair LECF2-LECR2, 46 of 48 *cag* positive strains (95.8%) gave positive amplicon. This may be due to some mismatch or deletion at the primer binding site. The promoter region of the *cagA* gene was found in all the *cag* positive strains. All the strains gave a positive amplicon with the primer set cag5cf & cag3cr showing that all the *cag* PAI positive strains were *cagA* positive.

**Partially deleted *cag* PAI among the Kolkata strains** - After a detailed analysis of the Kolkata strains, only 5 out of 48 (10.4 %) carried the *cag* PAI with partial deletion. Of these five partially deleted strains, three - I-110 (HP0535-HP0536) and I-338 (HP0530-HP0531), OSC36B (HP0532-HP0534) had deletion in two genes, which was confirmed by both Dot blot hybridization and PCR assay. In the rest two strains, San 61 and San73, multiple genes were deleted, and further in San 61, the Left end region was also deleted.

**Intact cag PAI among the Kolkata strains -** In this study, 92.3% (48 of 52) of the strains were cag PAI positive. Since the four cagA negative strains were also negative for all other genes tested, the cag PAI genes present in Kolkata strains were divided into two major types: intact-PAI and totally deleted -PAI genes. In this study using both PCR and Dot Blot, we have found that among the 48 strains that were positive for cagA, 43 strains (89.6 %) were found to carry the complete cag PAI while only 5 out of 48 (10.4 %) carried the cag PAI with partial deletion. Among the intact cag PAI, 24 of 26 (92.3%) cag PAI positive strains were from Duodenal Ulcer and 19 of 22 (86.4 %) were from Healthy volunteers. About 2 of 30 (7.7 %) of the Duodenal Ulcer patients and 3 of 22 (13.6%) among the Healthy volunteers had partially deleted cag PAI. This result showed that most of the Kolkata H. pylori strains has the capacity to cause disease as far as cag PAI is concerned.

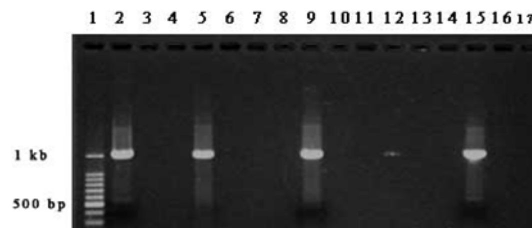


**Fig 8.1.1a:** Representative PCR result of HP0532-HP0534 gene using primer set PAInew2F-PAI25AS among the Kolkata strains. Lane 1-100 bp marker, lane 2-positive control, lanes 5, 9 and 15 produced positive amplicon; Lane 17-Negative control. By PCR only 3 out of 48 *cag*PAI positive strains yielded a positive amplicon.

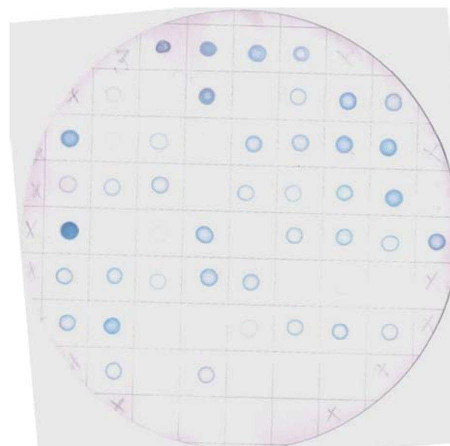

**Fig 8.1.1b:** Dot blot hybridization assay using non-radioactive DIG-labeled probe from 26695 generated by primer set PAInew2F-PAI25AS. 45 strains gave positive signals indicating the presence of target gene although 42 were negative by PCR.

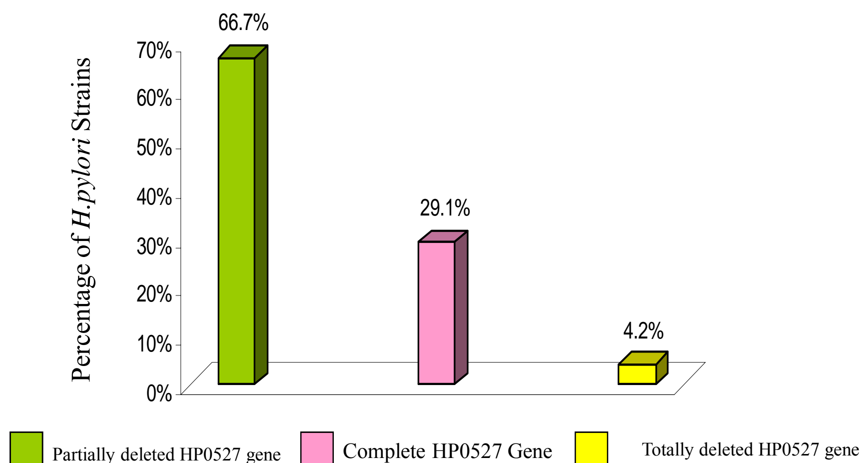


**Fig. 8.1.2:** Percentage of *H. pylori* strains having partially deleted, complete and totally deleted HP0527 gene of cag PAI among Kolkata strains. 66.7% of the strains gave a shorter amplicon, 29.1% of the strains gave same amplicon as that of 26695 while only 4.1% of the strains showed complete deletion of the gene.

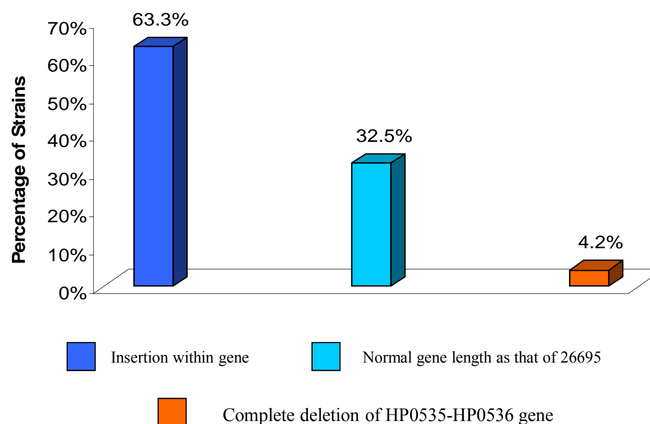


**Fig 8.1.3:** Graphical representation regarding variation in the gene length of HP0535-HP0536 gene using primer PAI27S-PAI27AS isolated from patients in Kolkata. About 63.3% of the Kolkata strains had insertion in the gene.

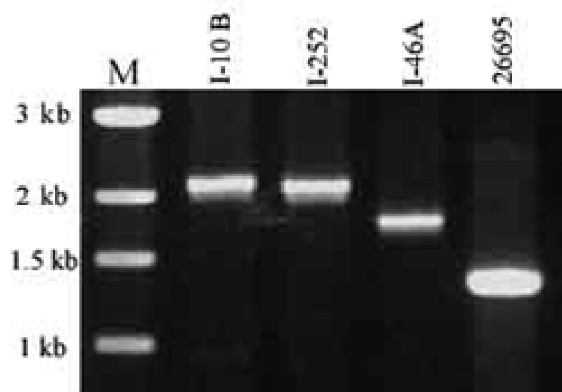


**Fig 8.1.4:** Representative PCR result of HP0535-536 gene using primer pair PAI27S-PAI27ASamong the Kolkata strains. Lane 1-1 kb marker and other lanes showing higher amplicon sizes as compared to 26695. Among 46 strains that amplified with the primer set , 31 of them produced a higher amplicon than that of 26695.

## 8.2 Correlation of histology with genotypes of *Helicobacter pylori* isolated from cases of Peptic ulcer, Non ulcer dyspepsia, Gastric carcinoma and Lymphoma

Investigator: D.R. Saha

*Helicobacter pylori*, a gram-ve microaerophilic bacterium causes a life long infection in over half of the world's human population. About 60-90% people in developing world acquire infection in early childhood and develop persistent infection, which lasts for decades unless treated with antibiotics. Although a major section of the *H. pylori* infected people remain asymptomatic, certain percentage develop peptic ulcer diseases and again few percentage in the long run may develop the dreadful disease gastric carcinoma. The bacteria have direct influence in the disease process though the actual pathology behind the disease is not clear.

This project was undertaken to determine the association and tissue response to *Helicobacter pylori* with different diseased conditions and in healthy volunteers (HVs) and to correlate the histologic findings with c*agA, vacA* and other virulent genetic pattern of the organism. Endoscopic biopsy samples were collected from fundus and antrum of the stomach from S.S.K.M hospital Kolkata Five bits of tissue were taken – one for Rapid urease test, two in Brucella broth with 15% glycerol for culture and two in buffered formalin for histopathological examination. To know, *H. pylori* infection in respect to our climatic condition a total of 124 individuals (50 duodenal ulcer and 74 healthy volunteers) were included in the study after proper counseling. Gastro endoscopic biopsy samples were collected from antrum and fundus and processed for rapid urease test, histology and culture. Characterization of genotypes of *H. pylori* strains was identified. Inflammatory cellular infiltration was studied with the activity of gastritis.

Chronic deep gastritis (moderate to severe) was detected in 76 % duodenal ulcer (du) patients and 59% healthy volunteers (hvs). Chronic atrophic gastritis was present among 60% du and 32% hvs. Ultrastructural study of the colonization ability of *H. pylori* among du and hvs are in progress.