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Original Research

Genetic variability in the repeat region of cytotoxin associated gene A of Indian *Helicobacter pylori* strains and its implication on various acid peptic disorders

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ABSTRACT

Infection with the *cytotoxin associated gene* A positive *H. pylori* strain significantly results in higher grades of gastric mucosal inflammation and has been suggested to modulate several host genes resulting in varied disease outcome. The size and the immunogenicity of CagA protein among the infected subjects varies and is believed to be affected by the variations in the 3' region of *cag*A gene. Therefore the present study was designed to investigate the genetic variability of *cag*A gene of Indian *H. pylori* isolates and assess its implication on Inducible Nitric Oxide Synthase expression and clinical outcome. Size of the *cag*A gene was assessed in 123 *H. pylori* isolates by polymerase chain reaction and level of iNOS expression in the gastric tissue was performed using immunofluorescence assay. Complete *cag*A gene was found in all the *H. pylori* screened. *H. pylori* with short *cag*A were predominant among gastric cancer subjects. iNOS expression was found to be high in patients with short sized *cag*A gene. In conclusion, we found that variation in the *cag*A gene was found to be significantly associated with iNOS expression and severity of Gastro Intestinal disease.

Keywords: *Helicobacter pylori*, *cag*A, variable size, iNOS.

INTRODUCTION

Helicobacter pylori is a gram –ve, spiral bacterium that primarily colonizes the human gut and causes diverse gastrointestinal (GI) diseases. The large difference in infection rates ranging from 10% in developed countries like USA as against 85% in developing countries like India [1, 2, 3], has sparked series of controversies of its precise relationship with the humans [4]. The main reason responsible for different clinical outcomes may be attributed partially to the highly plastic genome of *H. pylori* [5]. Several reports in the past have widely reported the role of principal virulence genes such as the *cag*A, *vac*A, *bab*A, *ice*A, *hrg*A, and *fla*A in the etiopathogenesis of various GI disorders [6, 7]. With the proven role of genes of the *cag* pathogenicity island (*cag*-PAI) that translocates the CagA protein via the type IV secretory system [5], it has become absolutely essential to study the contribution of individual genes of this ~40kb *cag*-PAI in disease pathogenesis. Of the 30 ORFS that comprise the pathogenicity island, *cag*A corresponding to *hp*0547 in the ATCC 26695 genome is the major player that secret-

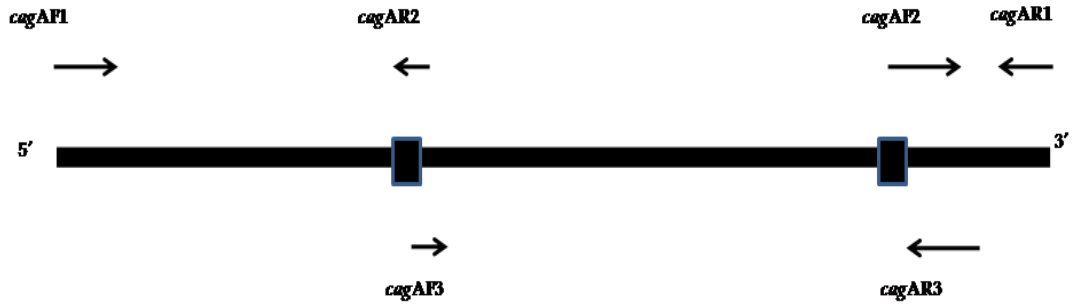

Figure 1. Schematic representation of PCR amplification of complete *cag*A gene of *Hp*

es the CagA protein (reference). The *cag*A gene of the intricate *cag*-PAI is the largest gene (~3.6kb) that encodes for a 120-145kDa immunodominant CagA protein, mainly characterized by the presence of stretch of conserved amino acids such as tyrosine phosphorrylation motifs (TPMs) and EPIYA motifs [8]. Upon translocation into the host epithelial cells, this CagA protein is known to trigger a complex cascade of events thereby leading to the destabilization of the cytoskeletal proteins [9]. Though number of studies throughout the world has underscored its role in disease manifestation, recent studies have attempted to understand the role of genetic variability on CagA translocation and disease outcome. Studies have elucidated that the 3' region of *cag*A is susceptible to genetic variations owing to the presence of variable number of repeats [10, 11]. Earlier reports have shown that CagA protein displays C-terminal variability to produce types and numbers of repeat sequences among different ethnic communities leading to diverse clinical outcomes [10-15, 26, 27]. The plasticity of *cag*A gene has also been reported to alter the expression of host factors. [16] However, their status and implications thereof in the Indian *H. pylori* have not been reported yet.

Inducible nitric oxide synthase (iNOS) or NOS-II, originally discovered in cytokine-induced macrophages is a high output enzyme, which produces toxic amounts of NO that represents an important component of the antimicrobial, antiparasitic and antineoplastic [17-19], activity of these cells. *H. pylori* infection is known to elicit a host inflammatory response with iNOS generation of NO to potentially eradicate the organism [20]. However, because of this bacterium's ability to persist, the inflammatory response becomes chronic and predisposes to cancer with persistence of iNOS expression. As the degree of gastric mucosal injury is a multifactorial event, that vastly depends on both bacterial and host factors. Therefore the present study was intended to assess the genetic diversity of 3' variable region of *cag*A among the Indian *H. pylori* strains and investigate the plausible relationship with iNOS expression.

EXPERIMENTAL PROCEDURES

A total of 123 well characterized *H. pylori* isolates from 123 subjects (males 52, females 71) with various GI disorders were studied. Genomic DNA isolation and histopathological analysis was performed as reported previously by us [2]. The study protocol was approved by Institutional Ethics Committee, Deccan College of Medical Sciences.

Genotyping of cagA gene of Hp

Amplification of the complete *cag*A gene was performed using specific oligonucleotide primers reported previously **(Figure 1)**[21]. Amplification included a positive and negative control (DNA from DH5α strain of *E. coli*) [2]. Amplification was performed as described previously [23], with minor modifications (annealing: 50^{o}C for 1min). After amplification, the amplimers were sequenced on both strands using a BigDye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, FosterCity, CA, USA) on an ABI PRISM 3730x*I* Genetic Analyzer (Applied Biosystems, Germany) as per the manufacturer's recommendation. Nucleotide sequences were aligned and analyzed using GENETYX-Mac version 11.2.3 (Software Development, Tokyo, Japan and compared with CPY3401 (AY121840) and J99 (AE001439.1) sequences published in NCBI database.

Histopathology

Section (4μM) from paraffin embedded biopsies (antrum and corpus) was stained with Haematoxylin & Eosin stain. To avoid bias, a single and experienced pathologist who was completely blinded to the clinical and molecular data performed histological assessment. Grading of gastritis was performed as per the updated Sydney system of classification [22].

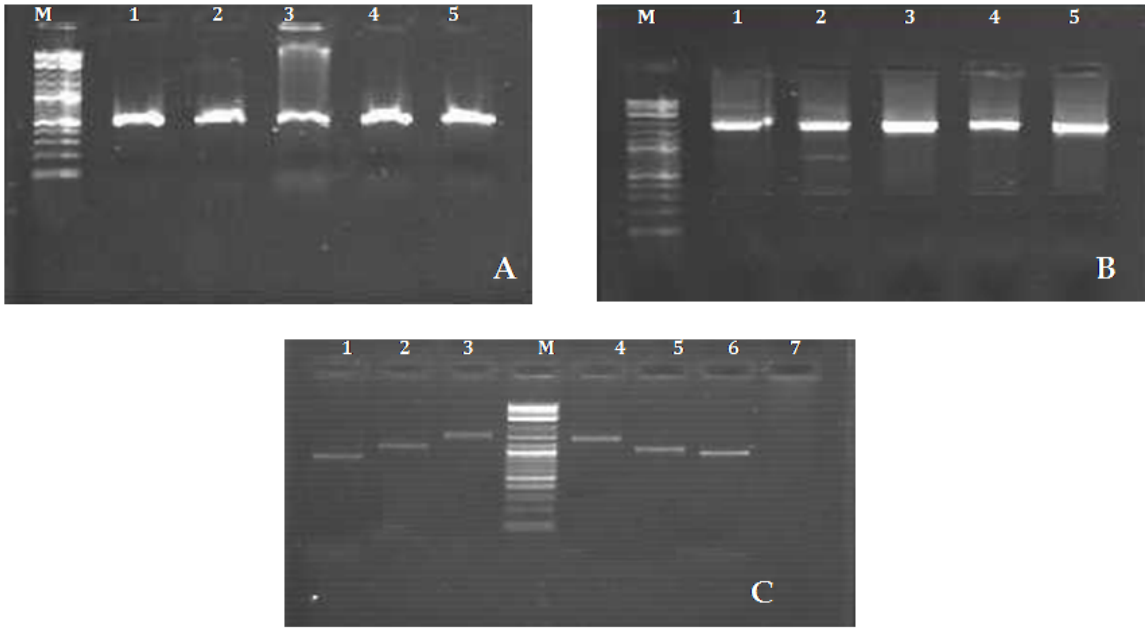

Figure 2. (A) Gel Image showing the 3' region of *cag*A gene of *Hp.* Lane 'M' shows the PCR marker, Lane 1, 2, 3 and 4, 5 shows PCR amplification from Overt and less overt gastric disorders.
(B) Gel Image showing the variable region of *cag*A gene of *Hp.* Lane 1 and 4 shows the PCR amplified with 970bp, Lane 2 and 5 shows 1100 bp and Lane 3 and 6 shows PCR amplification with 1300bp.
(C) Gel Image showing the 5' region of *cag*A gene of *Hp.* Lane 'M' shows the 100bp PCR marker, Lane 1, 2 and 3, 4, 5 shows PCR amplification from Less overt and Overt gastric disorders.

Expression of iNOS

Immunofluorescense for assessing the expression of iNOS was performed as described previously [23]. In brief, 5µm paraffin embedded sections were dewaxed and rehydrated by gradient alcohol treatment. The sections were then washed with sterile phosphate buffered saline (pH 7.0) for 10 min and non specific binding was blocked by 3% bovine serum albumin (BSA) for 30min. After blocking, 200µl goat polyclonal antibody anti-human iNOS (Santa Cruz Cat #SC-49058) was added and incubated at 37°C for 1hr. Sections were then washed thrice with 1X PBS after which 200µl donkey anti-goat IgG conjugated with TEXAS RED (Santa Cruz Cat #SC-2783) was added and incubated at 37°C in dark for 45min. Auto fluorescence was removed using auto florescence reducing agent. After thorough washing, sections were mounted in 4',6-Diamidino-2-phenylindole, HCl (DAPI) (Santa Cruz Cat #SC-3598) and 50% glycerol with anti fade component. Slides were then read with fluorescence microscope (Ziess AxioPlan-2) and images were captured using Axiovision software by multicolor digital camera. DAPI was captured with UV emission filter and TEXAS RED with long pass 610nm filters.

Statistical Analysis

The diversity of 3' variable region of *cag*A gene of *Hp* and its association with acid peptic disorders were analyzed by using a χ^2 test and Fisher's exact using SISA online software. Probability values (*p*) of <0.05 were considered as statistically significant.

RESULTS

The patients were broadly categorized into two major category based on the severity of the diseases: overt and less overt GI disorders**.** As clearly evident from the Table 1, all the 123 *Hp* isolates amplified with the specific primer (C1F/C1R) that spanned the complete length of *cag*A giving three different product sizes (3870, 3670, & 3540bp). The difference in the product size was due to variations seen in the 3' repeat region. Amplification with primers spanning the 5' (1980bp) and 3' region (490bp) was found in 102 (82.92%) and 107 (91.86%) respectively. **(Figure 2 A, B, C)** (Table-1) Albeit amplification with the 3' variable region was found in all the *Hp* isolates screened giving three different product sizes (1300, 1100, & 970bp). Based on the length of the variable fragment of *cag*A, *Hp* was classified as: *Hp* with

Table 1. Assessment of *cag*A gene of *H. pylori* in clinical isolates

Clinical groups	Status of the *cag*A gene of *Hp*					
	Complete *cag*A	3' Region	5' Region	Variable Region		
				Short Arm	Medium Arm	Long Arm
Overt gastric disease Gastric cancer (45)	45 (100%)	42(93.3%)	38 (84.4%)	37 (82.2%)	03 (6.6%)	05 (11.1%)
Prepyloric ulcer (24)	24 (100%)	18 (90.0%)	18 (90.0%)	02 (8.3%)	05 (20.8%)	17 (70.8%)
Duodenal ulcer (18)	18 (100%)	16(88.8%)	15 (83.3%)	02 (11.1%)	14 (77.7%)	02 (11.1%)
Total Overt gastric diseases (87)	**87 (100%)**	**76 (87.35%)**	**71 (81.6%)**	**41 (47.1%)**	**22 (25.2%)**	**24 (27.5%)**
Less overt gastric disease Non Ulcer dyspepsia (15)	15 (100%)	15 (100%)	15 (100%)	03 (20.0%)	06 (40.0%)	06 (40.0%)
Gastritis(21)	21(100%)	16(76.19%)	16(76.19)	02 (9.5%)	05 (19.04%)	14 (57.14%)
Total Less Overt gastric diseases (36)	**36 (91.6%)**	**31 (86.1%)**	**31 (86.1%)**	**05 (13.8%)**	**11 (30.55%)**	**20 (55.55%)**
Total (n=123)	**123(100%)**	**107 (91.86%)**	**102 (82.92%)**	**46 (37.39%)**	**33(26.82%)**	**44(35.77%)**

long *cag*A gene (3870), medium sized *cag*A (3670) and short sized *cag*A (3540).

With respect to the severity of the GI disease, we observed that *Hp* with short sized *cag*A was highly predominant among patients with overt than with less overt GI disease (p=0.001). When the size of *cag*A gene was extrapolated in individual disease condition, short sized *cag*A was found in gastric carcinoma subjects (82.2%), medium sized *cag*A predominated in duodenal ulcer subjects (77.7%) followed by subjects with NUD (40%), long sized *cag*A was found more among pre-pyloric ulcer patients (70.8%) followed by those with gastritis (57.14%) (Table-1). The accession numbers of the Gen Bank submissions of subset of the samples sequenced for the 3' variable region of the *cag*A gene of *Hp* are (FJ599712- FJ599743).

Histopathological assessment of subjects with long, medium & short *cag*A revealed that patients infected with short sized *cag*A had marked histological lesions: chronic gastritis- 6(13.3%), atrophy-2(4.4%), grade-II incomplete metaplasia- 2(4.4%), dysplasia in 1(2.2%) and gastric carcinoma in 34(75.5%) of the total 45 subjects. Subjects with medium sized *cag*A of *Hp* showed: chronic gastritis- 19(67.8%), atrophy- 3(10.7%), grade-II incomplete metaplasia- 3(10.7%), dysplasia in 2(7.1%) and gastric carcinoma in 1(3.6%). Similarly among subjects infected with long sized *cag*A- chronic gastritis- 6(13.3%), atrophy- 2(4.4%), grade-II income plete metaplasia- 2(4.4%), dysplasia in 1(2.2%) and gastric carcinoma in 34(75.5%).

Immunofluorescense demonstrated strong expression of iNOS among subjects infected with short sized *cag*A 28(62.2%) in comparison to those harboring long and medium sized *cag*A- 8(28.5%) and Mild to moderate intensity reflecting mild to moderate expression iNOS levels was observed among subjects with long to medium sized *cag*A **(Figure 3)**.

DISCUSSION

The highly plastic genome of the *H. pylori* is the major reason for the evolution of genotypically distinct strains of this pathogen worldwide. With vast difference in the prevalence rates it has been difficult to establish precise relationship that co-exists between *Hp* and diverse disease condition [10-15, 23, 24, 25]. Bacterial and host genetic factors are believed to play a major role in determining the clinical outcome [25]. Among multitudes of virulence factors, the *cag*A gene still remains the most widely studied gene owing to its central role in the type IV secretion system and the inflammation inducing genes [5]. Our study showed that infection with short *cag*A gene together with strong iNOS expression may increase the susceptibility of overt GI disease.

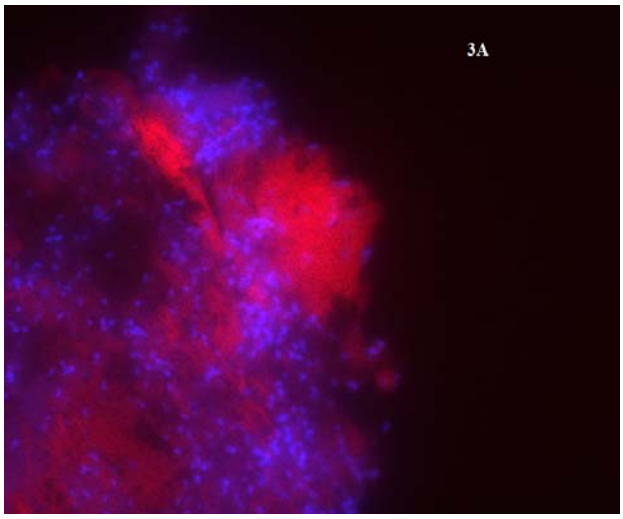

Figure 3A. Showing the Immunofluorscense of tissue level expression of iNOS with respect to the long arm region of *cag*A gene variable region of *Hp*.

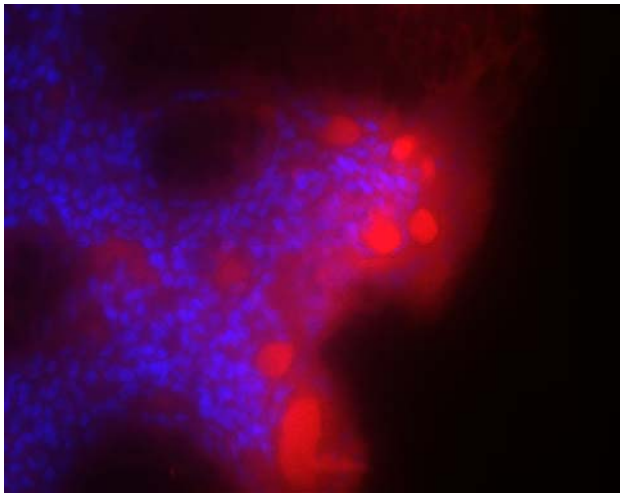

Figure 3B. Showing the Immunofluorscense of tissue level expression of iNOS with respect to the medium arm region of *cag*A gene variable region of *Hp*.

In the present study, we found complete *cag*A gene of *H. pylori* to be present in all the strains screened irrespective of the disease status. The variable region of *H. pylori* which causes variation in the size of the CagA protein resulted in three different sized *cag*A gene (long, medium & short). *H. pylori* with long *cag*A was commonly found in patients with pre-pyloric ulcer and gastritis, medium sized *cag*A-*H. pylori* were highly present in duodenal ulcer and non ulcer dyspeptic patients. On the contrary, gastric cancer patients (82.2%) were found to harbor *H. pylori* with short *cag*A gene. The results of

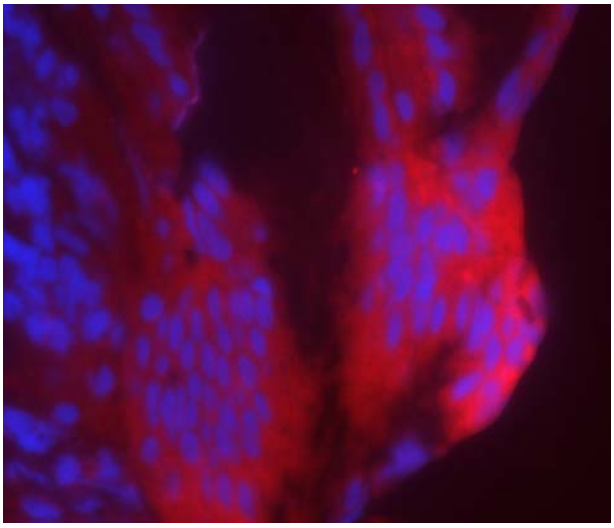

Figure 3C. Showing the Immunofluorscense of tissue level expression of iNOS with respect to the short arm region of *cag*A gene variable region of *Hp*.

histopathological analysis showed that precancerous lesions predominated among those infected with short sized *cag*A than those with long and medium sized *cag*A. Our findings are in contradiction to those reported by many authors [21, 10] which reported higher inflammatory scores besides higher atrophic and intestinal metaplasia index. The probable reason may be attributed to the genetic heterogeneity of the *H. pylori* which differs from one geographic region to another. Further the results obtained of the present study are also strengthened by the immunofluorescence findings which found that gastric tissues colonized with short sized *cag*A showed high expression levels of iNOS as compared to the counterparts with long- or medium sized *cag*A. However direct relation between the heightened iNOS expression and size of the *cag*A remains protracted as expression of iNOS is tissue specific affairs which differ in accordance to the degree of tissue damage [17].

Colonization of the gastric mucosa with type-I *H. pylori* (*cag*+ve) strains has been known to the deliver immunodominant CagA toxin that further damage and reprogram host cells [5]. In addition, infection with *cag*A+ve strains has been reported to modulate the expression of several host factors such as COX-2, iNOS, IL-1β, etc which together puts the host at risk for accumulating genetic and epigenetic changes thereby leading to the development overt GI disorders [17, 28]. The results of our study therefore support the findings that *H. pylori* related GI disease is a cumulative result of bacterial and host factors primarily driven by bacterial toxins.

In summary, the results of the study showed that *cag*A gene was unanimously present among all the cases. Variations in the *cag*A was found to be associated with both iNOS expression as well as severity of gastric disease thereby suggesting that bacterial and host factors interact as dangerous liaisons in triggering off a cascade of events resulting in variable disease outcome.

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Conflict of Interest

The authors have no conflict of interest to disclose.

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