

Original Research

Epidemiology and clinical importance of *Helicobacter pylori* genotypes in adults with various acid peptic disorders

¹Santosh K. Tiwari, ¹Manoj G, Sivaram G, ¹Saikant R, ¹Avinash Bardia, ¹Vishwas Sharma, ¹Md. Aejez Habeeb, ¹Zakia Abid, ^{*2}Aleem A. Khan, ^{*1}CM Habibullah¹

¹Center for Liver Research and Diagnostics, Deccan College of Medical Sciences, Kanchanbagh, Hyderabad, 500 058, Andhra Pradesh, INDIA

²Department of Pathology, Deccan College of Medical Sciences, Kanchanbagh, Hyderabad, 500 058, Andhra Pradesh, INDIA

Corresponding author

***Dr. Aleem A. Khan, Ph.D**, Scientist & Head, Center for Liver Research and Diagnostics, Deccan College of Medical Sciences, Kanchanbagh, Hyderabad, 500 058, Andhra Pradesh, INDIA.

Telephone & Fax: +91-40-24342954; Email: aleemkhan_clrd@rediffmail.com

ABSTRACT

Background & Aim: *Helicobacter pylori* infection plays a crucial role in the development of spectrum of gastrointestinal diseases including functional dyspepsia. Global efforts for complete eradication of *H. pylori* have been successful in some parts of the developed nations resulting in steep decline in their prevalence rates; it still touches stratospheric levels in most of the South East Asian countries. Though, reports have stratified well choreographed interplay of bacterial, host and environmental factors to determine disease outcome. Genotyping *H. pylori* based on the putative virulence genes greatly distinguishes highly pathogenic from those with less pathogenic strains. Hence, the present study aimed to evaluate the prevalence of genotypic variants of *H. pylori* in the study population and establish a causal role between genotypes and disease outcome.

Materials & methods: Prevalence and genotypes of *H. pylori* was evaluated in 360 patients with diverse gastric etiologies and the association of genotypes with severe and less severe gastric disorders was performed using multiplex amplification system.

Results: 66.7% of the subjects were assessed to possess active *H. pylori* infection as confirmed by 16S rRNA amplification with higher prevalence among males than in females. Analysis of genotypic data revealed genotype *cagT+ve/hrgA+ve/cagA+ve/cagE+ve/vacAs1+ve* to be most common in subjects with severe gastroduodenal diseases and other variants had higher predominance in clinically less overt disease conditions.

Conclusion: Genotypic data obtained implicates existence of strong association between highly virulent strains of *H. pylori* and clinical outcome.

KEYWORDS: *H. pylori*, prevalence, genotypes, gastroduodenal disease.

INTRODUCTION

Indian subcontinent is one of the richest sources of genetic studies with gene pool of one sixth of the global population. The genetic architecture of this subcontinent is highly complex, as each population is unique in their genetic composition; etiologies of diseases are often

different from other global populations¹, this is one of the primary reasons why major hypotheses fail tremendously in Indian context. The best classical example which can personify this stance is the etiopathogenesis of gastric pathogen *Helicobacter pylori* which undauntedly reigns

Table 1. Prevalence of *H. pylori* with respect to overtness of the gastrointestinal diseases

Clinical Status	Total No. of Subjects	Urease Positive (%)	Culture Positive (%)	16S rRNA Positive (%)	Histo-pathology (%)
Overt form of disease	284	178 (62.6)	162 (57.0)	190 (66.9) ^a	156 (54.9)
Less severe form of disease	76	46 (60.5)	33 (43.4)	50 (65.8) ^b	20 (26.3)
Total	360	224 (62.2)	195 (54.2)	240 (66.7)	176 (48.9)

^a 16S rRNA amplification vs. culture for diagnosing *H. pylori* in overt form of diseases, odd ratio=1.5222 ($p=0.0157$; 95% CI= 1.0824 to 2.1408).

^b 16S rRNA amplification vs. culture for diagnosing *H. pylori* in less overt form of diseases, odd ratio=2.5058 ($p=0.0061$; 95% CI= 1.3003 to 4.8289).

Note:

- Patients with gastric adenocarcinoma (GC), pre-pyloric ulcer (PPU), duodenal ulcer (DU) and peptic ulcer disease (PUD) were grouped into overt form of diseases.
- Patients presenting with gastroesophageal reflux disease (GERD) and non-ulcer dyspepsia (NUD) were grouped into less severe form of gastric disorders.

the stomach of significant proportion of world population leading to various gastroduodenal diseases². Though its plausible relationship with humans remains an enigma, the increasing number of reports asserts its role as a potential pathogen that inflicts severe mucosal damage rather than a bystander^{3,4,5}. Since its discovery, this pathogen has been associated with a spectrum of gastroduodenal disease ranging from chronic gastritis, peptic ulcer disease, gastric adenocarcinoma and mucosa associated lymphoid tissue (MALT) lymphoma⁶. Besides this, statistical data suggests *H. pylori* to be present in almost two-thirds of the population with prevalence as high as 75-90% in developing and underdeveloped countries and as low as 10% in developed nations^{7,8}. This has spurred considerable amount of interest among researchers to exemplify reasons for vast difference in the prevalence rates and unearth the molecular mechanisms by which *H. pylori* leads to different disease manifestations.

Intuition into the epidemiological aspects of this bacterium implicated socio-economic status and hygienic conditions to be the principle determinants responsible for the vast difference in prevalence rate⁷. Numerous epidemiological data from Indian sub-continent have already demonstrated an upsurge in the prevalence rate, the present study therefore attempts to update the current statistics^{7,8}. Further, analysis of array of data on *H. pylori* associated gastrointestinal diseases revealed various *H. pylori* virulence factors to play independent roles in the disease outcome in addition to other cofactors such as the host genetic and environmental factors⁹. This suggests that bacterial factors are equally responsible for varied clinical outcome.

Till date several criteria based on the different genes have been reported to evaluate the virulence trait of *H. pylori*, but the one based on the cytotoxin-associated gene A (*cagA*) is most widely accepted¹⁰. Scrupulous

analysis of large number of literatures revealed increased risk of peptic ulcer disease and gastric cancer in subjects with *cagA*+ve *H. pylori*^{10,12}. However, recent data from across the world have reported clinical outcome of *H. pylori* to be independent of the *cagA* status^{11,12}. This indicates involvement of other putative genes of *H. pylori* both within the ~40kb gene fragment *cag*-pathogenicity island (*cag*-PAI) and outside the PAI such as the vacuolating associated cytotoxin A (*vacA*), induced by contact with epithelium A (*iceA*), etc., in predicting the clinical outcome. Albeit many studies in this regard are reported,^{12,13,14} there is still dearth of confronting data which can provide valuable reason for the diverse clinical outcome.

Recent studies on *H. pylori* and virulence have been successful in demonstrating enhanced risk of overt form of gastroduodenal diseases among individuals infected with more virulent strain of *H. pylori* than those with less pathogenic strains^{15,16} and have reported higher predictive values of certain virulent genotypes in the development of severe disease manifestations. Some other studies have also implicated pivotal roles of other virulence factors viz., *cagE* (*hp0544*), *cagT* (*hp0532*), *vacA*, *babA* and *hrgA* in the etiology of these diseases^{2,14,15,17,18,19,20}. Previous studies on genotypes of *H. pylori* from Indian sub-continent reported common genotypes based on the genes of the *cag*-PAI in subjects with gastritis, peptic ulcer and few cases of gastric carcinoma^{21,22,23}. All these studies commonly indicate that pathogenic strains of *H. pylori* possess factors that differentiate it from weakly or non-virulent strains thereby suggesting that genotyping of *H. pylori* may be a useful strategy for determining clinical outcome of the disease. In addition, it has also been postulated that patient's vulnerability of developing overt form of gastric disorders increases with an increase in the number of virulence determinants possessed by *H. pylori* suggesting separate roles for each of them in causing

Table 2. *H. pylori* positivity with respect to various clinical categories.

Clinical Status	Total No. of Subjects	Urease Positive (%)	Culture Positive (%)	16S rRNA Positive (%)	Histopathology (%)
Gastric adenocarcinoma	110	68 (61.8)	64 (58.2)	75 (68.2)	58 (52.7)
Pre-pyloric ulcer	92	60 (65.2)	51 (55.4)	60 (65.2)	52 (56.5)
Duodenal ulcer	64	40 (62.5)	38 (59.8)	45 (70.3)	37 (57.8)
Peptic ulcer	18	10 (55.5)	09 (50.0)	10 (55.5)	09 (50.0)
GERD	29	18 (62.1)	13 (44.8)	20 (68.9)	08 (27.6)
NUD	47	28 (59.6)	20 (42.5)	30 (63.8)	12 (25.5)
Total	360	224(62.2) ^a	195(54.2) ^b	240(66.7)	176(48.9) ^c

^a16S rRNA amplification vs. urease, $p=0.0286$;

^b16S rRNA amplification vs. culture, $p= 0.0001$;

^c16S rRNA amplification vs. histopathology, $p= 0.0001$;

Fisher's exact test demonstrated the above data to be statistically significant ($p<0.05$).

histological abnormality. To support this hypothesis, recent study by Zambon, et al,¹⁵ reported worsened inflammatory scores and intestinal metaplasia among patients co-expressing *cagA*, *s1vacA*, *babA2* and *m1vacA* genes of *H. pylori* and also showed that these subset of patients were more prone to develop severe gastric disorders. Further, findings of the study confirmed that combination of different virulence genes rather than presence of a single gene fragment to be more ideal and pragmatic approach for genotyping *H. pylori* and identifying patients at highest risk of developing overt gastric disorders.

Therefore, the present study was carried out with an objective to evaluate the prevalence of *H. pylori* and determine the genotypic variants of *Helicobacter pylori* in the study population based on the *cagA*, *cagE*, *cagT*, *vacA* and *hrgA* genes with respect to their disease status.

Materials and methods

A total of 360 subjects (225 males and 135 females; age range of 21-55 years) undergoing upper gastrointestinal endoscopy at the Department of Gastroenterology and Hepatology, Deccan College of Medical Sciences and allied hospitals were included after taking informed consent in the study. Demographic data that included questionnaire on their birth place, family history, profession, diet, smoking and alcohol habits were recorded. Subjects with previous exposure to antibiotics, proton pump inhibitors, H2-blockers or regular intake of NSAIDs during the past two months were excluded from the study. The study protocol was approved by Institutional Ethics Committee.

Based on the clinical diagnosis, patients were categorized as follows: 110-carcinoma of the stomach (84 males and 26 females); 92-pre-pyloric ulcer (52 males and 40 females); 64- duodenal ulcer (36 males and 28 females); 18-peptic ulcer disease (14 males and 4 females); 29-gastro esophageal reflux disease (15 males

and 14 females) and 47 had non-ulcer dyspepsia (24 males and 23 females). Four gastric biopsies, 3 from the antral and one from the corpus region of the stomach were collected during endoscopy. Two antral biopsy specimens collected in phosphate buffered saline (PBS) and brucella broth (Difco Chemicals, Detroit, USA) supplemented with 2% fetal calf serum (FCS) were utilized for genomic DNA isolation and culturing *H. pylori* respectively. The remaining two (one each from the antrum and corpus) was collected in 10% buffered formalin for histopathological studies. In patients with malignant tumors, 2 biopsies were taken from the tumor area in 10% buffered formalin for histopathological analysis and remaining two biopsies were collected from the unaffected area for the isolation of live culture and genomic DNA isolation respectively.

Diagnosis of *H. pylori* was confirmed by culture, 16S rRNA PCR amplification and histopathology. Subjects negative for all the three tests were considered as negative for *H. pylori*. Molecular diagnosis and genotyping of *H. pylori* was performed using multiplex PCR assay previously reported by us⁶. Histopathological assessment for the presence of *H. pylori* was scored according to the updated Sydney system of classification²⁴.

Statistical Analysis

Statistical analysis was performed using StatistiXL (version 1.8) software (StatistiXL, Nedlands, Western Australia) <http://www.statistixl.com/downloads/download.asp>, Microsoft EXCEL, SISA ([http:// www.quantitativeskills.com/sisa/statistics/fisher.htm](http://www.quantitativeskills.com/sisa/statistics/fisher.htm)), SPSS (version 11) soft- ware (SPSS Inc., Chicago, IL, USA). The probability level (p) <0.05 was used as significance criterion. Student *t*-test has been used in many cases. Analysis of variance (ANOVA) *F*-test was performed to compare continuous variables between the groups using ORIGIN (version 5.0) statistical software (OriginLab,

Table 3. Prevalence of *H. pylori* infection among patients with severe and less severe form of gastrointestinal diseases with respect to sex.

Clinical Category	Males			Females		
	N	Positive	Negative	N	Positive	Negative
Overt form of disease	186	137 (73.70) ^a	49 (26.30)	98	53 (54.10)	45 (45.90)
Less Overt form of disease	39	27 (69.20) ^b	12 (30.80)	37	23 (62.20)	14 (37.80)
Total	225	164 (72.90)	61 (27.10)	135	76 (56.30)	59 (43.70)

^a*H. pylori* in males with overt disease vs. females with overt disease, odds ratio=2.3739 ($p=0.0010$; 95% CI=1.4197 to 3.9692). Statistically highly significant.

^b*H. pylori* in males with less overt disease vs. females with less overt disease, odds ratio=1.3696 ($p=0.5167$; 95% CI=0.5293 to 3.5438). Statistically not significant.

Table 4. Prevalence of *H. pylori* infection in various gastrointestinal diseases with respect to sex.

Clinical Category	Males			Females		
	N	Positive	Negative	N	Positive	Negative
Gastric adenocarcinoma	84	61 (72.6)	23 (27.4)	26	14 (53.8)	12 (46.1)
Pre-pyloric ulcer	52	40 (76.9)	12 (23.1)	40	20 (50.0)	20 (50.0)
Duodenal ulcer	36	28 (77.80)	08 (22.20)	28	17 (60.7)	11 (39.3)
Peptic ulcer disease	14	08 (57.1)	06 (42.9)	04	02 (50.0)	02 (50.0)
GERD	15	09 (60.0)	06 (40.0)	14	11 (78.60) ^b	03 (21.4)
NUD	24	18 (75.0)	06 (25.0)	23	12 (52.2)	11 (47.8)
Total	225	164 (72.9) ^a	61 (27.1)	135	76 (56.3)	59 (43.7)

^a*H. pylori* positivity in males vs. females, $p=0.0005$; statistically highly significant (Fisher's exact test).

^b*H. pylori* positivity in females with GERD vs. males, $p=0.1819$; data not statistically significant (Fisher's exact test).

Corporation, Northampton MA 01060, United States). The Fisher's exact test which calculates an exact probability value for the relationship between two dichotomous variables was used to check the prevalence of *H. pylori* infection among patients with severe and less severe form of gastrointestinal diseases with respect to sex.

Results and discussion

The results corresponding to various aspects of the study are tabulated from Table 1 to Table 9.

The discovery of *Helicobacter pylori* has revolutionized the perception about the etiology of gastroduodenal diseases, where it is thought to play a central role in vast majority of subjects. One principal effect of this discovery has been to trivialize the role of psychosocial factors in the genesis of these gastric disorders. The epidemiological, clinical and genetic evidence strongly suggests a complex and well orchestrated interaction between the host, bacterial and environmental factors to be mainly responsible in determining disease outcome²⁵. Though multitude of host and bacterial factors has already been reported that determines person's susceptibility to develop a particular gastric disorder, these reports do not divulge much information in settings

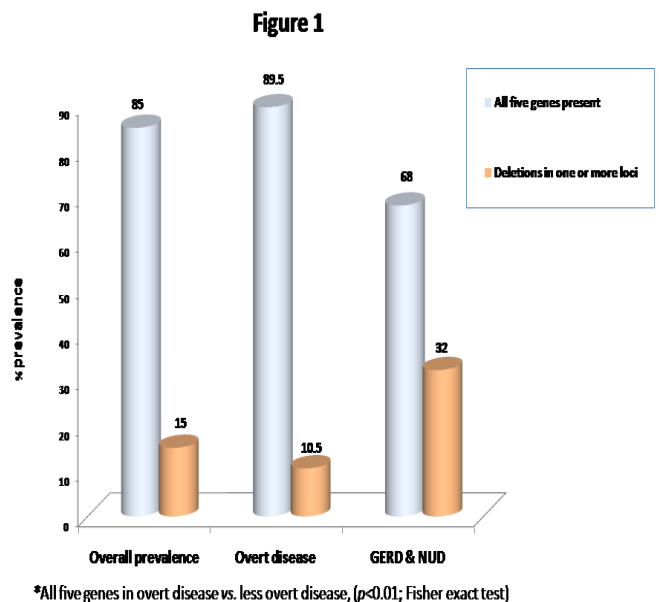


Figure 1. Depicts overall combined prevalence of five virulence genes of *H. pylori* and among subjects with overt and less severe gastrointestinal diseases.

Table 5. Distribution of five virulence genes of *H. pylori* in patients with various gastrointestinal diseases.

Genes	Gastric Carcinoma (n=75)	Duodenal Ulcer (n=45)	Pre-pyloric Ulcer (n=60)	Peptic Ulcer Disease (n=10)	GERD (n=20)	NUD (n=30)	Total (n=240)
<i>cagT</i>	75 (100)	44 (97.8)	60 (100)	10 (100)	20 (100)	26 (86.7)	235(97.9)
<i>hrgA</i>	75 (100)	45 (100)	60 (100)	10 (100)	20 (100)	30 (100)	240(100)
<i>cagA</i>	68 (90.7)	38 (84.4)	58 (96.7)	06 (60.0)	12 (60)	22 (73.3)	204(85)
<i>Cage</i>	75 (100)	41 (91.1)	60 (100)	06 (60.0)	19 (95.0)	23 (76.7)	224(93.3)
<i>vacA</i>							
s1 allele	67 (89.3)	29 (64.4)	56 (93.3)	05 (50.0)	13 (65.0)	16 (53.3)	186(77.5)
s2 allele	8 (10.7)	16 (35.6)	04 (6.7)	05 (50.0)	7 (35)	14 (46.7)	54(22.5)

$p < 0.0001$ (one-way ANOVA).

Table 6. Distribution of target genes of *H. pylori* with respect to overtness of the disease presentation.

Genes	Overt Disease (n=190)	Less severe form of disease (n=50)
<i>hrgA</i>	190 (100)	50 (100)
<i>cagT</i>	189 (99.4)	46 (92)
<i>cagE</i>	182 (95.7)	42(84)
<i>cagA</i>	170 (89.4)	34 (68)
s1 allele of <i>vacA</i>	157 (82.6)	29 (58)
s2 allele of <i>vacA</i>	33 (17.4)	21 (42)

^a Presence of all the five genes in overt category vs. less severe gastric disorder, $p < 0.05$ (Mann Whitney two-tailed test).

Note:

- Patients with gastric adenocarcinoma (GC), pre-pyloric ulcer (PPU), duodenal ulcer (DU) and peptic ulcer disease (PUD) were grouped into overt form of diseases.
- Patients presenting with gastroesophageal reflux disease (GERD) and non-ulcer dyspepsia (NUD) were grouped into less severe form of gastric disorders.

where this bacterium is highly prevalent. Hence, the present study determined the frequency of *H. pylori* infection and various genotypes of *H. pylori* prevalent in subjects with diverse gastroduodenal diseases.

Though *H. pylori* is world's commonest chronic bacterial infection, marked differences exist in prevalence rates between different countries and between different communities or regions within the same country. Developed countries have lower prevalence rates compared to developing countries,²⁶ this difference appears to be related to the socioeconomic status of the population^{7, 8, 27}. In the present study, 66.7% of the total patients were found infected with *H. pylori* which appears to be parallel to those seen in developing countries. Comparison of *H. pylori* prevalence in overt and less severe form of gastrointestinal disease, showed 66.9% and 65.8% respectively (Table 1). Many studies from India report prevalence of *H. pylori* as approximately 65-90% in patients with duodenal ulcer, gastric ulcer and gastric cancer and 50-70% in patients with NUD²⁸. On the contrary studies from the west have depicted 50-65% prevalence in patients suffering from non-ulcer dyspepsia^{29,30} and 80-90% in patients DU^{31,32}. These results clearly demonstrate a strong association of

H. pylori infection in the pathogenesis of various gastric disorders.

It has been documented that prevalence of *H. pylori* among males is relatively high on par with females³³. In this study, sex-wise analysis of the prevalence showed that males had an increased frequency of *H. pylori* positivity (72.9%) when compared to females (56.3%) (Table 3) as observed by others³³. Further, when the sex-wise prevalence of *H. pylori* was correlated with the overt nature of the disease, it was found that males presenting with overt form of disease had 2.3739 folds increased frequency of *H. pylori* infection compared to females with severe form of gastrointestinal disorders (Table 3). The frequency of *H. pylori* infection among male subjects (69.2%) with less overt form of disease was comparable with those in females (62.2%). When prevalence among males and females was compared with the individual disease status, higher rates of prevalence were observed in males. The data clearly indicates that 72.6% males with gastric adenocarcinoma harbored *H. pylori* in comparison to 53.8% of females, similarly male subjects with pre-pyloric ulcer, duodenal ulcer, peptic ulcer and NUD had prevalence of 76.9%, 77.8%, 57.1% and 75% respectively in comparison to 50%, 60.7%, 50% and 52.2% among females presenting with similar

Table 7. Depicts combined prevalence of all the five target genes in subjects with various gastric conditions.

Clinical Status (n)	All five genes present (%) ^a	Deletions in one or more loci (%) ^a
Gastric carcinoma (75)	68 (90.7)	07(9.3)
Duodenal ulcer (45)	38 (84.4)	07(15.6)
Pre-pyloric ulcer (60)	58 (96.7)	02(3.3)
Peptic ulcer disease (10)	06 (60)	04(40)
GERD (20)	12 (60)	08(40)
NUD (30)	20 (66.7)	10(33.3)

^a Student's *t*-test (two-tailed) showed $p=0.098$, data not significant.

Table 8. Genotypes of *H. pylori* among patients with various gastrointestinal diseases.

Genotypes	Gastric Carcinoma (n=75)	Duodenal Ulcer (n=45)	Pre-pyloric Ulcer (n=60)	Peptic Ulcer Disease (n=10)	GERD (n=20)	NUD (n=30)
<i>cagT</i> +ve/ <i>hrgA</i> +ve/ <i>cagA</i> +ve/ <i>cagE</i> +ve/ <i>vacAs1</i> +ve	65 (86.7)	28 (62.2)	56 (93.4)	05 (50.0)	07 (35.0)	16 (53.3)
<i>cagT</i> +ve/ <i>hrgA</i> +ve/ <i>cagA</i> +ve/ <i>cagE</i> +ve/ <i>vacAs2</i> +ve	03 (4.0)	10 (22.2) ^a	02 (3.3)	1 (10.0)	05 (25.0)	06 (20.0)
<i>cagT</i> +ve/ <i>hrgA</i> +ve/ <i>cagA</i> -ve/ <i>cagE</i> -ve/ <i>vacAs2</i> +ve	0	03 (6.7)	0	04 (40.0)	01 (5.0)	03 (10.0)
<i>cagT</i> -ve/ <i>hrgA</i> +ve/ <i>cagA</i> -ve/ <i>cagE</i> -ve/ <i>vacAs2</i> +ve	0	01 (2.2)	0	0	0	04 (13.4)
<i>cagT</i> +ve/ <i>hrgA</i> +ve/ <i>cagA</i> -ve/ <i>cagE</i> +ve/ <i>vacAs1</i> +ve	02 (2.6)	02 (4.4)	0	0	06 (30.0)	0
<i>cagT</i> +ve/ <i>hrgA</i> +ve/ <i>cagA</i> -ve/ <i>cagE</i> +ve/ <i>vacAs2</i> +ve	05 (6.7)	01 (2.2)	02 (3.3)	0	01 (5.0)	01 (3.3)

^a Genotype *cagT*+ve/*hrgA*+ve/*cagA*+ve/*cagE*+ve/*vacAs2*+ve in DU subjects vs. pre-pyloric ulcer subjects, highly significant (relative risk=7.3333; $p=0.0009$, 95% CI=2.2669 to 23.7231).

disease conditions (Table 4). These findings of the study are similar to those reported previously³⁴. The possible reason for this could be that environmental factors predispose men to have greater risk for both *H. pylori* infection and various gastrointestinal diseases than females³⁴. Secondly, higher gastric acid secretory status has been reported to play favoring role since it has been shown that men have a higher gastric acid secretory capacity than women of similar age and weight³⁵. Perhaps this could be one of the reasons for predominance of infection in males as observed in this study. However, the present study noticed females with GERD had higher rates of infection on par with males (78.6% vs. 60%). Albeit statistically not significant ($p>0.05$), the reason for this finding warrants further investigation in a large population.

The accurate diagnosis of *H. pylori* has been a crux ever since the discovery of this gastric pathogen. Even though there are number of both invasive and non-invasive methods in practice, they mainly differ in differential rates of detection^{36,37}. The present study however reports higher rates of detection of *H. pylori* by DNA PCR compared to urease, culture and histopathology. This study found that 16S rRNA PCR, which targets and amplifies the most conserved gene, was able to detect *H. pylori* in 240 (66.7%) subjects of

the total subjects screened compared to 62.2% by urease, 54.2% by culture and 48.9% by histopathology (Table 1 & 2).

H. pylori exhibit high degree of diversity at both the genotypic and phenotypic levels. Henceforth, the present study used a PCR based genotypic system previously reported by us,⁶ for identification and genotyping strains of *H. pylori*. The results obtained demonstrated *hrgA* gene amplified in all the samples followed by *cagT* (97.9%), *cagE* (93.3%) and *cagA* (85%). All the 240 subjects studied were *vacA* positive, with s1 allele present in 77.5% and s2 in 22.5% respectively (Table 5). A similar trend was observed in the prevalence of these virulence genes with respect to overtness of the gastric disorders (Table 6) which clearly indicates all the genes namely *cagT*, *cagE*, and *cagA* to be predominantly present in subjects with severe gastric disorders than those with less severe disease. The s1 subtype of *vacA* gene predominated among subjects with overt disease such as pre-pyloric ulcer, gastric carcinoma, duodenal ulcer and peptic ulcer (Table 6) compared to those with GERD and functional dyspepsia (NUD). As evident from the data, none of the patients harbored multiple strains of *H. pylori* infection. When the prevalence of these genes was compared with respect to individual disease status, the *hrgA* and *cagA* genes were present in 100% subjects

Table 9. Genotypes of *H. pylori* infection among patients with severe and less severe form of gastrointestinal diseases.

Genotypes	(n=240)	% of Genotypes	Overt disease (%)	Less severe form of disease (%)
<i>cagT+ve/hrgA+ve/cagA+ve/cagE+ve/vacAs1+ve</i>	177	73.70	154 (87) ^a	23 (13)
<i>cagT+ve/hrgA+ve/cagA+ve/cagE+ve/vacAs2+ve</i>	27	11.30	16 (59.3)	11 (40.7)
<i>cagT+ve/hrgA+ve/cagA-ve/cagE-ve/vacAs2+ve</i>	11	4.50	07 (63.6)	04 (36.4)
<i>cagT-ve/hrgA+ve/cagA-ve/cagE-ve/vacAs2+ve</i>	05	2.10	01 (20)	04 (80)
<i>cagT+ve/hrgA+ve/cagA-ve/cagE+ve/vacAs1+ve</i>	10	4.20	04 (40)	06 (60)
<i>cagT+ve/hrgA+ve/cagA-ve/cagE+ve/vacAs2+ve</i>	10	4.20	08 (80)	02 (20)

^a *cagT+ve/hrgA+ve/cagA+ve/cagE+ve/vacAs1+ve* vs. other genotypes, data highly significant ($p<0.001$; Fisher's exact test).

with gastric carcinoma, pre-pyloric ulcer, peptic ulcer and GERD whereas subjects with duodenal ulcer and NUD demonstrated almost equal prevalence i. e. 97.8% and 97.9% respectively. With reference to the clinical status, *vacAs1* allele was prominent in 93.3%, 89.3%, 64.4% and 50% of patients with pre-pyloric ulcer, gastric carcinoma and duodenal ulcer and peptic ulcer compared to those 65% with GERD and 53.3% with NUD respectively. The data obtained affectively demonstrates that all the target genes except *cagE* and *s1* allele of *vacA* showed no association with the disease status when prevalence of individual genes was compared with the clinico-pathologic conditions, thereby confirming the hypothesis that disease outcome does not depend on the presence or absence of a single virulent determinant rather it is a result of combined presence of various virulence genes co-expressing in *H. pylori*. These results are in concert with those reported earlier by Louw et al,³⁸ and Maeda et al,³⁹ who independently demonstrated no correlation between the presence of virulence genes and disease outcome.

When combination of all the target genes was assessed, all five genes were found to be present in 85% whereas remaining 15% cases had deletions in one or more genes (Figure 1).

In general, presence of all five genes increased the susceptibility of the subject to develop severe gastric diseases than those, which lacked one or more loci (85% vs. 15%). With respect to severity of the disease, it was observed that 89.5% subjects with overt gastric presentations possessed all the five genes present compared to 68% with less severe form of gastric disorders ($p<0.01$). On the contrary, deletions in one or more loci were predominant in less severe disease pathologies compared to overt conditions (32% vs. 10.5%). Similar trend was observed with reference to the individual disease categories, deletions were more commonly observed among subjects presenting with esophageal reflux and non-ulcer dyspepsia and their occurrence was rare in subjects with pre-pyloric ulcer and gastric carcinoma

(Table 7). This data effectively exemplifies the importance of virulence markers of *H. pylori* in causation of severe disease manifestations.

Comparison of the genotypic data obtained in the present study with the clinical course, depicted higher prevalence of the genotype *cagT+ve/hrgA+ve/cagA+ve/cagE+ve/vacAs1+ve* among patients with pre-pyloric ulcer (93.4%) and gastric carcinoma (86.7%) followed by duodenal ulcer subjects (62.2%) and other presentations (Table 8). Overall, this genotype was present in 73.7% of the total subjects analyzed with higher occurrence among patients with overt presentations i.e. those with ulceration and gastric carcinoma (87% vs. 13%) than with GERD and NUD respectively (Table 9). The genotype *cagT-ve/hrgA+ve/cagA-ve/cagE-ve/vacAs2+ve* was observed to be least (2.1%) prevalent in our study (Table 9). The results of the present study are in concert with those obtained by Koehler et al,¹⁶ which demonstrated strong correlation between the presence of *cagA* gene & *vacAs1* subtype and also showed 4.2 folds increased prevalence of the *s1* allele of *vacA* in gastric adenocarcinoma. The results of the present study are also in accordance with those reported by Zambon et al,¹⁵ that reported higher risk of developing intestinal metaplasia in subjects infected with virulent *H. pylori* which possessed more number of virulence determinants, thereby underscoring the importance of individual genes in disease causation. Another important finding of this study is that *hrgA* gene was detected in all the patients irrespective of their clinical condition. This finding of the study are similar to those previously reported by our group⁴⁰ but markedly differs from those obtained by Ando et al,¹⁹ which reported predominance of *hrgA* among *H. pylori* isolated from gastric cancer patients of Asian origin. The reason for this discordant result needs further evaluation in large number of *H. pylori* isolates from different geographical areas.

In conclusion, the observations of this study demonstrate prevalence of *H. pylori* in our population to be similar to those observed by others. Also, a strong

association was observed between overt gastrointestinal disease and specific genotypes of *H. pylori*. The study also substantiates that *H. pylori* strains co-expressing combination of major putative virulence genes differed in their toxigenicity in comparison to less toxigenic strains lacking either a fragment of gene or expressing less toxic allele. Though, the present study did not probe in detail about the impact of *H. pylori* genotypes and molecular changes pertinent to each of them which is one of the limitation of the study, it outlines the importance of genotypes in clinical outcome. Further, it also provides impetus for the forthcoming studies which can successfully be planned to demonstrate the precise pathway by which these genotypes alter gastric physiology thereby leading to diverse gastric diseases. This strategy may therefore enable us to identify ulcerogenic/carcinogenic *H. pylori* strains in near future.

References

1. Kivisild T, Roosti S, Metspalu M, et al. The genetic heritage of the earliest settlers persists both in Indian Tribal and Caste Population. *Am J Hum Genet.* 2003; 72: 313-332.
2. Fischbach W, ChanAO, Wong BCY. *Helicobacter pylori* and gastric malignancy. *Helicobacter* 10(Suppl 1). 2005; 34-39.
3. Crowe SE. *Helicobacter* Infection, chronic Inflammation, and the development of malignancy. *Curr Opin Gastroenterol.* 2005; 21(1): 32-38.
4. Ahmed N. 23 years of the discovery of *Helicobacter pylori*: Is the debate over?. *Annals of Clinical Microbiol and Antimicrobials.* 2005; 4:17
5. Martin JB and John CA. *Helicobacter pylori* persistence: biology and disease. *J Clin Invest.* 2004; 113(3): 321–333.
6. Tiwari SK, Khan AA, Manoj G, et al. A simple multiplex PCR assay for diagnosing virulent *Helicobacter pylori* infection in human gastric biopsy specimens from subjects with gastric carcinoma and other gastro-duodenal diseases. *J Appl Microbiol.* 2007; 103(6): 2353-2360.
7. Ahmed KS, Khan AA, Ahmed I, et al. Prevalence study to elucidate the transmission pathways of *Helicobacter pylori* at oral and gastroduodenal sites of a South Indian population, *Singapore Med J.* 2006; 47(4): 291-295.
8. Ahmed KS, Khan AA, Ahmed I, et al. Impact of household hygiene and water source on the prevalence and transmission of *Helicobacter pylori*: a South Indian perspective. *Singapore Med J.* 2007; 48(6): 543-549.
9. Tiwari SK, Manoj G, Vasanth Kumar G, et al. Prognostic significance of genotyping *Helicobacter pylori* infection in patients in younger age groups with gastric cancer. *Postgrad Med J.* 2008; 84(990): 193-197.
10. Shimoyama T, Fukuda S, Tanaka M, et al. CagA seropositivity associated with development of gastric cancer in a Japanese population. *J Clin Pathol.* 1998; 51: 225-228.
11. Held M, Engstrand L, Hansson LE, et al. Is the association between *H. pylori* and gastric cancer confined to CagA-positive strains?, *Helicobacter.* 2004 ; 9: 271-277.
12. Yamaoka Y, Kodama T, Gutierrez O, et al. Relationship between *Helicobacter pylori* iceA, cagA, and vacA status and clinical outcome: studies in four different countries. *J Clin Microbiol.* 1999; 37(7): 2274-2279.
13. van Doorn LJ, Figueriedo C, Sanna R, et al. Clinical relevance of the cagA, vacA, and iceA status of *Helicobacter pylori*. *Gastroenterol.* 1998; 115: 58-66.
14. Yamaoka Y, Kodama T, Kita M, et al. Relationship of vacA genotypes of *Helicobacter pylori* to cagA status, cytotoxin production, and clinical outcome. *Helicobacter.* 1998; 4: 241-253.
15. Zambon CF, Navaglia F, Basso D, et al. *Helicobacter pylori* babA2, cagA, and s1 vacA genes work synergistically in causing intestinal metaplasia. *J Clin Pathol.* 2003; 56: 287-291.
16. Koehler CI, Mues MB, Dienes HP, et al. *Helicobacter pylori* genotyping in gastric adenocarcinoma and MALT lymphoma by multiplex PCR analyses of paraffin wax embedded tissues. *Mol Pathol.* 2003; 56: 36–42.
17. Xiaobo L, Wenzhong L, Weiwen X, et al. Clinical implications and prevalence of cagA, cagE and cagT genes in the pathogenicity island of *Helicobacter pylori* strains isolated from Shanghai patients. *Chinese J Digestive Dis.* 2001; 2(3): 133-136.
18. Fujimoto S, Ojo OO, Arnqvist A, et al. *Helicobacter pylori* BabA expression, gastric mucosal injury, and clinical outcome. *Clinical Gastroenterol Hepatol.* 2003; 5(1): 49–58.
19. Ando T, Wassenaar M, Peek RM et al. A *Helicobacter pylori* Restriction Endonuclease-replacing gene, hrgA, is associated with gastric cancer in Asian strains. *Cancer Res.* 2002; 62: 2385-2389.
20. Ando T, Aras RA, Kusugami K, et al. Evolutionary history of hrgA, which replaces the restriction gene hpyIII in the hpyIII locus of *Helicobacter pylori*. *J Bacteriol.* 2003; 185: 295-301.
21. Tiwari SK, Aleem AK, Shakeel AK, et al. PCR based analysis of the cag-PAI of *Helicobacter pylori* from saliva: An approach for rapid molecular genotyping in correlation with disease status. *J Gastroenterol Hepatol.* 2005; 20(10): 1560-1568.
22. Ali M, Khan AA, Tiwari SK, et al. Association between cag-pathogenicity island in *Helicobacter pylori* isolates from peptic ulcer, gastric carcinoma, and non ulcer dyspepsia subjects with histological

- changes. *World J Gastroenterol.* 2005; 11(43): 6815-6822.
23. Kauser F, Khan AA, Hussain MA, et al. The *cag* pathogenicity island of *Helicobacter pylori* is disrupted in the majority of patient isolates from different human populations. *J Clin Microbiol.* 2004; 42: 5302-5308.
 24. Dixon MF, Genta RM, Yardley JH, et al. Classification and grading of gastritis: the updated Sydney System. *Am J Surg Pathol.* 1996; 20: 1161-1181.
 25. Melmed RN and Gelpin Y. Duodenal ulcer: the helicobacterization of a psychomatic disease? *Isr J Med Sci.* 1996; 32 (3-4): 211-216.
 26. Goh KL. *Helicobacter pylori* infection in Asia. Epidemiology of *Helicobacter pylori* infection, peptic ulcer and gastric cancer: Experience in a multiracial South East Asian country. *Helicobacter Res.* 2000; 4(1): 34-40.
 27. Graham DY, Adam E, Reddy GT, et al. Seroepidemiology of *H. pylori* infection in India. Comparison of developing and developed countries. *Dig Dis Sci.* 1991; 36: 1084-1088.
 28. Abraham P and Bhatia SJ. First national workshop on *Helicobacter pylori*: position paper on *Helicobacter pylori* in India. *Indian J Gastroenterol.* 1997; 16: S29-S33.
 29. Petross CW, Appleman MD, Cohen H, et al. Prevalence of *Campylobacter pylori* and association with antral mucosal histology in subjects with and without upper gastrointestinal symptoms. *Dig Dis Sci.* 1988; 33: 649-653.
 30. Bernersen B, Johnsen R, Bostad L, et al. Is *Helicobacter pylori* the cause of dyspepsia? *Br Med J.* 1992; 304: 1276-1279.
 31. Tytgat GNJ, Noach JA, Rauws EAJ. *Helicobacter pylori* infection and duodenal ulcer disease, *Gastroenterol Clin North Am.* 1993; 22: 127-138.
 32. Fiocca R, Vilani L, Luinetti O, et al. *Helicobacter* colonization and histopathological profile of chronic gastritis in patients with or without dyspepsia, mucosal erosion and peptic ulcer. A morphological approach to the study of ulcerogenesis in man, *Virchows Arch A, (Pathol Anat.)* 1992; 420:489-498.
 33. Replogle ML, Glaser SL, Hiatt RA, et al. Biologic sex as a risk factor for *Helicobacter pylori* infection in healthy young adults. *Am J Epidemiol.* 1995; 142(8): 856-863.
 34. Vakiland BJ and Mulekar AM. Studies with the maximal histamine test. *Gut.* 1965; 6: 364-371.
 35. Chen W, Shu D, Chadwick VS. *Helicobacter pylori* infection in interleukin-4-deficient and transgenic mice, *Scand J Gastroenterol.* 1999; 34: 987-992.
 36. Anonymous. Technical annex: tests used to assess *Helicobacter pylori* infection. Working party of the European *Helicobacter pylori* Study Group, *Gut* .1997; 41: S10-18.
 37. Campbell M and Machin D. Medical statistics: a common sense approach, In John Wiley & Sons Ltd, West Sussex, England. 1993; 36-38.
 38. Louw JA, Kidd MSG, Kummer AF, et al. The relationship between *Helicobacter pylori* infection, the virulence genotypes of the infecting strain and gastric cancer in the African setting, *Helicobacter.* 2001; 6: 268-273.
 39. Maeda S, Ogura K, Yoshida H, et al. Major virulence factors, *vacA* and *cagA*, are commonly positive in *Helicobacter pylori* isolates in Japan *Gut* . 1998; 42: 338-343.
 40. Manoj G, Tiwari SK, Sharma V, et al. Association of *Helicobacter pylori* restriction endonuclease-replacing gene, *hrgA* with overt gastrointestinal diseases. *Arq de Gastroenterol.* 2008; 45(3):225-229.