Virulence attributes of *Helicobacter pylori* isolates & their association with gastroduodenal disease

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Background & objectives: Certain genotype(s) of *Helicobacter pylori* strains may play important role in the development of gastric cancer (GC) and peptic ulcer disease (PUD). This study was undertaken to investigate the association of *cagA*, *cagA3/* region subtypes, *babA2* and *vacA* genotypes of *H. pylori* with GC, PUD and non-ulcer dyspepsia (NUD) as there are no such studies from India.

Methods: A total of 348 consecutive adult patients (NUD 241, PUD 45, GC 62) undergoing upper gastrointestinal endoscopy between September 2002 and May 2007 in a tertiary referral centre at Lucknow, north India, were enrolled. *H. pylori* infection was diagnosed by rapid urease test, culture, histopathology and PCR. Genotyping for *cagA*, *cagA3/* subtypes, *babA2* and *vacA* was performed by PCR using sequence specific primers.

Results: *H. pylori* infection was higher in patients with PUD than with GC (80 vs. 56.5%, *P* < 0.01) and NUD (80 vs. 55.2%, *P*= 0.002). *cagA* positive *H. pylori* isolates were detected in 80 per cent in GC, 83.3 per cent in PUD and 76.7 per cent in NUD with no significant difference among them. Only A subtype of *cagA3/* was detected and its distribution in GC, PUD and NUD was 68.8, 69.4 and 52.6 per cent respectively. Presence of *babA2* genotype was 31.4 per cent and it had significant association with PUD when compared with NUD (52.8 vs. 26.3%, *P*<0.003). On univariate regression analysis, s1a allele was associated with GC (*P*<0.050) and s1a/m2 *vacA* genotype with both GC (*P*=0.014) and PUD (*P*=0.016).

Interpretation & conclusions: *H. pylori* infection was strongly associated with PUD with a very high proportion of patients with GC have s1a allele and s1a/m2 *vacA* genotype. Both s1a/m2 *vacA* genotype and *babA2* are associated with PUD. The study shows that different virulence attributes of *H. pylori* are involved in different gastroduodenal disorders.

Helicobacter pylori has been identified as a major cause of peptic ulcer disease (PUD) and a risk factor for gastric cancer (GC) and mucosa-associated lymphoid tissue (MALT) lymphoma[1,2] . On a global scale, GC is the second commonest cancer in the world. There is substantial international variation in GC incidence with the highest rates reported from China, Japan and other Eastern Asian countries. Epidemiological studies have proved that *H. pylori* infection is considered as a risk factor for GC, and WHO International Agency

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for Research on Cancer has classified this bacterium as a definite carcinogen[2]. While the majority of the *H. pylori* infected individuals develop no significant clinical disease, some develop two kinds of divergent clinical diseases, PUD and GC[3]. The reasons for this may be related to differences in genetic susceptibility of the host, environmental factors, and genetic diversity of *H. pylori*[4]. In this context, the relevance of specific *H. pylori* virulence associated genes has been extensively studied. The cytotoxin associated gene A (*cagA)* was the first to be identified; about 60-70 per cent of *H. pylori* strains in the West were found to be *cagA+* and these strains were associated with duodenal ulcer (DU) and GC[5,6]. However, more than 90 per cent of *H. pylori* strains in Asia are *cagA+* irrespective of DU and GC[7,8]. The *cagA* gene can be classified into type A, B, C and D based on its 3′- terminal repetitive sequences[8], however, the association of these subtypes with clinical disease remains unclear. The vacuolating toxin (*vacA*) was subsequently discovered and its allelic variants were identified in the signal region (s1a, s1b, s1c or s2) and mid region (m1 or m2)[9]. Specifically, *vacA* s1/m1 strains have higher cytotoxic activity than s1/m2 strains, whereas s2/m2 strains have no cytotoxic activity[9]. Specific *vacA* genotypes are associated with level of toxin production and clinical diseases like PUD and GC in different part of the world[9-10]. Studies have also provided evidence that bacterial adherence factors may also contribute to the pathogenicity of *H. pylori*. The blood group antigen binding adhesin (*babA*) has been shown to mediate adherence of *H. pylori* to human blood group antigens on gastric epithelial cells[11]. However, the role of *babA2* gene in the development of GC and PUD remains undefined. It is well established that differential *cagA* and *vacA* genetic characteristics exist in *H. pylori* strains isolated from different geographical regions. Therefore, using molecular techniques to study the association of *H. pylori* genotypes or strains with gastroduodenal diseases has become an important study area. The current study was done with an objective to identify the frequency *cagA*, *cagA3′* region subtypes, *babA2* and *vacA* genotypes of *H. pylori* isolates and their association with gastroduodenal diseases.

Material & Methods

Study population: A total of 348 consecutive adult patients [62 GC, 45 PUD and 241 non ulcer dyspepsia (NUD)] who underwent upper gastrointestinal endoscopy at a tertiary referral center in northern India (Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow) between September 2002 and May 2007 were enrolled in this study. The diagnosis of gastroduodenal diseases was based on clinical, endoscopic and histopathological examinations. Patients with NUD were considered as controls. The ethics committee of the institute granted approval for the study protocol and the written consent was obtained from all the patients. Subjects who had received anti-microbial therapy, H_2 receptor blockers, proton pump inhibitors and non-steroidal anti-inflammatory drugs in the preceding 30 days prior to endoscopy or anti-*H. pylori* treatment in the past were excluded.

Detection of H. pylori infection: During each endoscopy, five antral biopsies were obtained and subjected to the following tests: one biopsy for rapid urease test (RUT), one for culture, two for histopathology and one for DNA extraction and *ureA* PCR following the standard protocol as described earlier[12]. *H. pylori* infection was diagnosed if any of the above tests was positive.

Histopathology: Sections of 5 μm were cut from formalin fixed biopsy, and embedded in paraffin wax. The sections were stained with hematoxylin and eosin for light microscopy. Patients with GC were confirmed by histopathology and classified into intestinal, diffuse and mixed according to the Lauren classification[13]. The different histological characteristics in patients with PUD and NUD were graded according to the updated Sydney system[14].

H. pylori genotyping: DNA was isolated from the bacterial growth and *H. pylori* positive gastric tissues using the QIAamp DNA mini kit (QIAGEN, Hilden, Germany) as per the manufacturer's instruction and subjected to PCR for the presence of *cagA*. Further, the detection of *cagA* 3′ region subtypes, *babA2* and *vacA* genotypes of *H. pylori* was carried out by PCR using specific primers (Table I). PCR was performed in a 50 μl reaction volume containing 100 ng of genomic DNA, 1 X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM each deoxynucleotide, 0.5 μM each specific primer and 1.25U of Taq DNA polymerase. PCR cycles for *cagA* and *vacA* genotypes were as follows: predenaturation at 94^0C for 5 min before adding Taq DNA polymerase; 27 cycles of 94^0C for 30 sec, 52^0C for 30 sec and 70^0C for 30 sec. PCR cycles for *cagA* 3′ region subtypes and *babA2* genotypes were as follows: 35 cycles of 94^0C for 1 min, 52^0C for 1 min and 72^0C for 1.30 min. Final extension was given at 72^0C for 8 min. All the PCR reactions were performed using thermocycler (Perkin Elmer Cetus, USA). The amplified product was electrophoresed in 2 per cent agarose containing

0.5 µg/ml ethidium bromide and examined under transluminator (UVS Systems, Hammond, USA).

Statistical analysis: The data analysis was performed by SPSS software (Version 12.0, SPSS, Chicago, IL, USA). The association of *H. pylori* status in relation to gastroduodenal diseases was performed by the Chi-square test. Univariate logistic regression analysis was used to analyze various *H. pylori* genotypes with gastroduodenal disease. *P* less than 0.05 was considered significant.

Results

A total of 348 patients (mean age: 46.78 ± 15.96 yr; 216 male) were enrolled and their distributions were as follows: GC 62 (mean age: 56.60 ± 15.42 yr; 47 male), PUD 45 (mean age: 49.47 ± 17.22 yr; 31 male) and NUD 241 (mean age: 43.75 ± 14.76 yr; 138 male).

Occurrence of *H. pylori* infection in our study population was 204 (58.6%). *H. pylori* infection was significantly higher in patients with PUD than with GC (80 vs 56.5%, $P<0.01$) and NUD (80 vs 55.2%, $P<0.002$). The distribution of different types of GC was as follows: intestinal type 25 (40.3%), diffuse type 37 (59.7%). None of the patients had mixed type of adenocarcinoma. As per the diagnostic criteria, 35 (56.5%) of 62 patients with GC had *H. pylori* infection. *H. pylori* infection was detected in significantly higher proportion in intestinal type than diffuse type of adenocarcinoma (18/25, 72% vs 17/37, 45.9%; $P<0.05$).

The histopathological characteristics of the gastric mucosa in *H. pylori* positive patients in PUD and NUD are shown in Table II. As per our diagnostic criteria,

Table I. Primer sequence for *H. pylori* genotypes

Region	Nucleotide sequence	Product size (bp)	Reference
cagA	5′-AGACAACTTGAGCGAGAAAG -3′ 5′-TATTGGGATTCTTGGAGGCG -3′	320	5
cagA 3′ region	5′-ACCCTAGTCGGTAATGGGTTA-3′ 5′-GTAATTGTCTAGTTTCGC-3′	642-756	8
babA2	5′-AATCCAAAAAGGAGAAAAAGTATGAAA-3′ 5′-TGTTAGTGATTTCGGTGTAGGACA-3′	850	29
s1a	5′-GTCAGCATCACACCGCAAC-3′ 5′-CTGCTTGAATGCGCCAAAC-3′	190	12
s1b	5′-AGCGCCATACCGCAAGAG-3′ 5′-CTGCTTGAATGCGCCAAAC-3′	187	12
s1c	5′-CTGCTTGAATGCGCCAAAC-3′ 5′-CTYGCTTTAGTRGGGYTA-3′	213	12
s2	5′-GCTTAACACGCCAAATGATCC-3′ 5′-CTGCTTGAATGCGCCAAAC-3′	199	12
m1	5′-GGTCAAAATGCGGTCATGG-3′ 5′-CCATTGGTACCTGTAGAAAC-3′	290	12
m2	5′-GGAGCCCCAGGAAACATTG-3′ 5′-CATAACTAGCGCCTTGCAC-3′	352	12

Table II. Histological findings in *H. pylori* infected patients with peptic ulcer disease (PUD) and non-ulcer dyspepsia (NUD)

Parameters	NUD (n=133)				PUD (n=36)			
A. Grading	None	Mild	Moderate	Severe	None	Mild	Moderate	Severe
H. pylori density	35	65	26	7	7	15	13	1
Neutrophil polymorphs	43	42	45	3	7	13	15	1
Chronic inflammation	30	49	36	18	1	18	14	3
B. Ungrading	NUD (n=133)				PUD (n=36)			
Foveolar hyperplasia	24				13			
Degenerative changes	65				17			
Lymphoid follicles	20				2			
Gastric atrophy	11				3			
Intestinal metaplasia	15				5			
Dysplasia	0				1			

133 (55.2%) patients with NUD and 36 (80%) patients with PUD had *H. pylori* infection.

Presence of *cagA*, *cagA* 3ʹ subtypes, *babA2* and *vacA* genotypes of *H. pylori* were determined on DNA extracted from *H. pylori* cultures/biopsies by PCR. Data were also compared between culture positive and negative *H. pylori* isolates (Table III). Overall the *cagA* presence in *H. pylori* isolates was 160/204 (78.4%); 28 (80%) of 35 isolates from GC, 30 (83.3%) of 36 from PUD, and 102 (76.7%) of 133 strains from NUD. The *cagA* was detected in almost equal proportions in *H. pylori* isolates from all the groups with no significant difference among the groups (Table IV and V).

Among four subtypes (A, B, C and D) of *cagA* 3ʹ region, only subtype A was detected in 58.3 per cent (119/204) of *H. pylori* isolates, remaining; 41.7 per cent were untypeable for all four subtypes. Presence of subtype A in GC, PUD and NUD was 68.6 (24/35), 69.4 (25/36) and 52.6 per cent (70/133), respectively. Although the subtype A of *cagA* 3ʹ region was higher both in GC and PUD, the difference was not significant (Table V).

babA2 genotype was present in only 31.4 per cent (64/204) of *H. pylori* isolates. Presence of *babA2* in GC, PUD and NUD was 28.6 (10/35), 52.8 (19/36) and 26.3 per cent (35/133), respectively (Table III). *babA2* genotype was significantly associated with PUD (*P*=0.003) and not with GC (*P*=0.789) (Table V).

Presence of *vacA* gene in our *H. pylori* isolates was 89.2 per cent (182/204) and the frequency in different groups was as follows: GC 94.3 (33/35), PUD 97.2 (35/36) and NUD 85.7 per cent (114/133). Mixed infection was detected in 3 (8.5%) and 5 (4.4%) patients with PUD and NUD, respectively. None of the patients with GC had mixed infection.

The overall distributions of different signal sequence alleles in our patient populations were as follows: s1a 126 (69%), s1b 2 (1.09%), s2 44 (24.17%), s1a/s1b 3 (1.6%), s1a/s2 3 (1.6%), s1a/s1b/s2 2 (1.09%) and untypeable 2 (1.09%). The distribution of

Table III. Comparison of genotypes between *H. pylori* culture positive strains and *H. pylori* culture negative gastric biopsies

Genotypes	Culture positive n=50 (%)	Culture negative n=154 (%)	Total n=204 (%)
cagA	40 (72)	120 (77.9)	160 (78.4)
babA	19 (38)	45 (29.2)	64 (31.4)
cagA 3ʹ region	27 (54)	92 (59.7)	119 (58.3)
vacA	45 (90)	137 (88.9)	182 (89.2)

Table IV. *cagA* and *babA2* genotypes of *H. pylori* in patients with gastric cancer (GC), peptic ulcer disease (PUD) and non-ulcer dyspepsia (NUD)

Group	Disease	*cagA* No. +ve/ No. investigated (%)	*babA2* No. +ve/ No. investigated (%)
I	GC	28/35 (80)	10/35 (28.6)
II	PUD	30/36 (83.3)	19/36 (52.8)
III	NUD	102/133 (76.7)	35/133 (26.3)
	Total	160/204 (78.4)	64/204 (31.4)

Table V. Association between *H. pylori* genotypes with gastric cancer and peptic ulcer disease by univariate regression analysis

Disease	*H. pylori* genotypes	*P* value	Odds ratio	95% CI
GC	*cagA*	0.678	1.216	0.484 - 3.052
	cagA3ʹ type A	0.094	1.964	0.891 - 4.330
	babA2	0.789	1.120	0.489 - 2.565
PUD	*cagA*	0.395	1.520	0.579 -3.986
	cagA3ʹ type A	0.075	2.045	0.931 - 4.492
	babA2	0.003	3.129	1.464 - 6.689

Table VI. Association between *H. pylori vacA* alleles/genotypes with gastric cancer by univariate regression analysis

Alleles/genotypes	Gastric cancer			Peptic ulcer disease		
	P value	Odds ratio	95% CI	*P* value	Odds ratio	95% CI
s1a	0.050	2.800	1.001 - 7.829[*]	0.072	2.417	0.924 - 6.321[*]
s1b	-	-	-	0.345	2.044	0.463 - 9.020
s2	0.024	0.276	0.090 - 0.842[†]	0.138	0.500	0.200 - 1.249
m1	0.811	0.850	0.225 - 3.211	0.542	1.417	0.462 - 4.341
m2	0.691	1.209	0.474 - 3.087	0.578	1.302	0.513 - 3.306
s1am1	0.859	1.161	0.223 - 6.043	0.918	1.091	0.210 - 5.664
s1am2	0.014	2.841	1.240 - 6.510[*]	0.016	2.695	1.207 - 6.019[*]
s2m2	0.029	0.189	0.043 - 0.840[†]	0.044	0.275	0.078 - 0.965[†]
s2m1	0.730	0.681	0.077 - 6.044	0.690	0.641	0.072 - 5.679

[*]Positive and [†]negative associations with gastric cancer

signal sequence in different groups of patients was as follows: in GC (n=33)- s1a 28 (84.8%), s2 04 (12.1%), untypeable 01 (3%); in PUD (n=35)- s1a 27 (77.1%), s2 05 (14.3%), s1a/s1b 01 (2.9%), s1b/s2 01 (2.9%), s1a/s1b/s2 01 (2.9%), and in NUD (n=114)- s1a 71 (62.3%), s1b 02 (1.8%) s2 35 (30.7%), s1a/s1b 02 (1.8%), s1a/s2 02 (1.8%), s1a/s1b/s2 01 (0.9%), untypeable 01 (0.9%). Majority of isolates were either s1a or s2. Out of 126 s1a positive isolates, 107 (82.6%) were found to be *cagA* positive and out of 44 s2 positive isolates, 29 (65.9%) were positive for *cagA*.

Overall, the most prevalent genotype of *vacA* was s1a/m2 in our patients as compared to s1a/m1, s2/m2 and s2/m1. Univariate logistic regression analysis showed that s1a allele was associated with gastric cancer (*P*=0.050) and s1a/m2 *vacA* genotype was associated with both gastric cancer (*P*=0.014) and PUD (*P* = 0.016) (Table VI), while s2/m2 genotype was detected in significantly higher proportion from NUD.

Discussion

The presence of *H. pylori* in patients with GC in our study was low and had no association with GC. Other tissue based study from India also failed to show an association between *H. pylori* infection and GC[15].

A high occurrence of *cagA* was seen in *H. pylori* isolates in the present study; 80 per cent in GC, 83.3 per cent in PUD 76.7 per cent in NUD. No correlation of *cagA* could be established with GC and PUD, although *cagA* had been identified as a virulence marker and associated with increased severity of disease in some geographic regions[16]. High prevalence of *cagA* (80-90%) independent of the disease status had been reported in other Indian studies as well[17,18]. These studies indicate that *cagA* cannot be considered as the sole virulence marker for determination of the disease outcome at least in India as has been reported from other geographic regions[16]. It is possible that some other genes of *cagA*-PAI are responsible for pathogenicity and disease outcome.

Since allelic variation in *cagA* exists and distinct *H. pylori cagA* subtype(s) may circulate in different regions[17], differences in *cagA* subtypes may provide a marker for differences in virulence among *cagA*-positive *H. pylori* strains. Yamaoka *et al*[8] reported the different structural subtypes of the *cagA* 3′ region (A to D) and found that 6 of the 7 type C were present in patients with GC in comparison to non cancer patients[8]. Overall, subtype A had been more frequently detected

in patients with different gastroduodenal diseases including GC[7,8]. Subtype A was the most frequent type encountered in Brazil (81%)[7]. Among four types (A, B, C and D) of *cagA* 3′ region, only A subtype was found in our study population that was neither associated with GC nor with PUD. The present study suggests that *cagA* 3′ region variants are not involved in gastric carcinogenesis and PUD. A total of 41.7 per cent isolates were found untypable with selected primers.

There is inconsistency in the prevalence and association of *babA2* genotype with the gastric diseases. However we reported 31.4 per cent prevalence of *babA2* in our population and found significant association with PUD when compared with NUD (*P*=0.003). Studies from China and four European countries reported the prevalence of *babA2* 25 and 30 - 60 per cent, respectively and found no correlation with clinical disease[19,20]. Recently, a study from Thailand reported 92 per cent positivity of *babA2* gene and found no significant association with clinical outcome[21]. However, a significant association of *babA2* genotype with duodenal ulcer (DU) and GC was observed in Brazil and Germany[22,23].

We showed that s1a allele had significant association with GC and it was more frequently present in patients with PUD. The prevalence of *vacAs2* strain in our population was approximately 25 per cent, which is similar to our previous report of 27 per cent in children[24], but is discordant with other Indian studies where the prevalence of *vacAs2* strain ranged from 2 to 10 per cent[17,25]. We also found very high number of *cagA* positive *vacAs2* strain (65.9%) in our patient population. This is the first study that reports high prevalence of *cagA* positive in *vacAs2* strains. These differences may be geographical and because of high frequency of *cagA* positive strains in our population with no significant difference between disease and control populations. Excluding the mixed infection and combining the groups, we observed that *vacA* s1a/m2 was frequently found in our study population, which is similar to several other findings[26]. Univariate regression analysis in our patients showed that s1a/m2 *vacA* genotype was associated with both gastric cancer (*P*=0.014) and PUD (*P*=0.016). These findings are markedly different from those reported association of s1a/m1 *vacA* genotype with PUD and GC[27,28].

In contrary to this, few studies had found no correlation of *vacA* genotypes with clinical outcome. However, Bulent *et al* found that the presence of *vacA*

genotype was not a predictive marker for peptic ulcer and non-ulcer dyspepsia[28].

In conclusion, *H. pylori* infection is strongly associated with PUD. *cagA* and *cagA3ʹ* region subtypes of *H. pylori* have no association with GC and PUD in the study. A significantly higher proportion of patients with GC have s1a allele and s1a/m2 *vacA* genotype. Besides s1a/m2 *vacA* genotype, *babA2* is also associated with PUD. The study suggests that different virulence attributes of *H. pylori* are involved in differential outcome of gastric disorders.

Acknowledgment

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