

Prevalence, diversity and disease association of *Helicobacter pylori* in dyspeptic patients from Pakistan

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Abstract

Introduction: The etiological association of *Helicobacter pylori* with gastric ulcer (GU), gastric cancer (GC), and duodenal ulcer (DU) is well-known. Understanding the epidemiology of *H. pylori* facilitates the estimation of disease burden in a certain population. This study presents the diversity of *H. pylori* genotypes and their association with different clinical outcomes among dyspeptic patients in Pakistan over a period of four years.

Methodology: Gastric biopsy samples from a total of 450 dyspeptic individuals were subjected to PCR, genotyping and histology.

Results: A total of 201 (45%) cases were found positive for *H. pylori*. The detection rate was high in GU (91%), DU (86%) and GC (83%) cases compared with those cases who had intact gastric mucosa (18%). Histology revealed the presence of infection in 68% of cases of mild/chronic nonspecific gastritis with others belonging to the GU sequel. *cagA* gene carriage was observed in 104 (51%) cases or mostly from DU, GU and GC groups, of which 97 were Western type strains while 3 were East-Asian type strains that are rarely observed in South Asia. *vacA* allelic variant s1am1 was most commonly observed, followed by s1am2, and s1bm1, with direct correlation in diseased cases (gastritis, GU, DU and GC). Prevalent genotypic combinations were s1am1/*cagA*⁻ in gastritis and s1am1/*cagA*⁺ in DU, GU, and GC.

Conclusions: Our study indicates the predominant circulation of Western type *cagA* and *vacA*s1am1 type *H. pylori* strains in Pakistan.

Key words: *H. pylori*; *cagA*; *vacA*; duodenal ulcer; gastric ulcer; Pakistan

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Introduction

Helicobacter pylori are associated with various gastrointestinal disorders such as gastritis, gastric ulcer (GU), and duodenal ulcer (DU) [1,2]. In last two decades, it has been associated with gastric adenocarcinoma (GC) [3-7], and it is now defined a class I carcinogen by the World Health Organization (WHO).

H. pylori carries various virulence factors, such as vacuolating cytotoxin A (VacA), CagA cytotoxin and sialic acid binding adhesin (SabA), that are implicated in the development of pathological conditions of gastric mucosa [9]. CagA is encoded by the cytotoxin associated gene A (*cagA*) present in the *Cag* pathogenicity island [10,11]. *H. pylori* strains can be

grouped as Western and East Asian subtypes on the basis of polymorphism in the 3' repeat region of the *cagA* gene [12]. Variable numbers of repeat sequences in each subtype vary the size of protein product and subsequently affect the pathogenicity of *cagA*⁺ *H. pylori* strains. The East Asian subtype of the *cagA* gene is more commonly associated with severe histological damage, *e.g.*, acute gastritis and gastric cancer, than Western subtypes [13-16]. Each subtype is prevalent in its respective geographical location; *i.e.*, East Asian type *cagA* is prevalent in East Asia and Western types are prevalent in North America and Europe [17]. Thus prevalence is considered both as an effective assessment tool to analyze the epidemiology

of the region and also as a marker to track human migration.

The VacA toxin encoded by the *vacA* gene is another virulence factor of *H. pylori* that induces cytoplasmic vacuoles formation and mitochondrial damage to gastric epithelial cells [18]. Pleomorphic combinations of signal (s) and middle (m) regions of the *vacA* gene affect the amount of vacuolating activity. Due to variable toxin activity, different *vacA* genotypes have been associated with different histological conditions [11] and are considered molecular markers of disease progression. Among all genotypic combinations, s1m1 and s1bm1 are considered to be the most virulent and are frequently reported in patients with acute gastritis [19], peptic ulcer [20], and GC [21], followed by s1m2, which is responsible for moderate toxin activity mainly due to the binding capacity of the m2 region with restricted cell types [22]. Although s2m1 and s2m2 are rare (especially s2m1) and are considered the least toxic of the genotypic combinations because of their inability to form vacuoles, they have been reported in patients with DU and GU [20,23].

Pakistan is considered among the countries with a high rate of gastroduodenal pathologies, especially DU; however, unlike the East Asian rim, the frequency of GC is low [24]. Several studies reported the high rate of *H. pylori* infection in the country based on serological, bacteriological, and histological observations [25-27]. Recent reports, however, document the prevalence of the *vacA* s1m1 genotype in Pakistan, endorsing the common trend of other South Asian neighbors [28,29]. The presence of the organism in drinking water has also been reported, indicating greater chances of infection for the community [30,31]. This study presents a four-year long assessment of *H. pylori* infection, prevalence of *cagA* and *vacA* genotypes, and their relatedness with clinical disease outcome among high-risk patients in Karachi, Pakistan.

Methodology

Patients

A total of 450 dyspeptic patients who underwent gastroduodenal endoscopy at a tertiary care hospital in Karachi were enrolled for the study. Recruitment was conducted from March 2005 to November 2008. Formal written consent was taken from every patient at the time of sample collection. Only patients with no previous therapy with anti-*Helicobacter* (antibiotic and proton pump inhibitor) and anti-cancer drugs and no history of immediate international migration were

included in the study. A questionnaire-based approach was used to generate background clinical and social information, while personal interviews were conducted with those patients who had difficulties completing the questionnaire. Patients were grouped on the basis of the gastric endoscopic appearance as “normal” in cases of intact mucosa, gastric ulcer (GU), duodenal ulcer (DU), gastritis, and gastric cancer (GC). The study was conducted with approval from the ethical review board, University of Karachi, Pakistan.

Sample collection and processing

Three gastric biopsy specimens (two from antrum and one from fundus) were collected from each patient and kept in sterile 20% glucose solution [32] for DNA extraction and in 10% formaldehyde (Scharlau, Barcelona, Spain) for histology. Samples were immediately transported to the laboratory in a light-proof insulated ice box and processed within an hour. After homogenization in sterile water, DNA was extracted using the SDS-PK method as described previously [33]. Samples were stored at -20°C. PCR analysis for the human β -globulin housekeeping gene was performed to check the quality of DNA.

Histology

Histology was conducted on 166 samples from patients who had damaged gastric mucosa and who were positive for *H. pylori* by PCR. Samples from normal (n = 26) and GC cases (n = 6) were not included because of endoscopically declared intact mucosa and extremely diverse gastric pathology respectively. The 3 μ m paraffin-embedded sections of biopsy samples were cut and stained with hematoxylin and eosin (H and E) for histological examination. The Sydney system was applied for histological slide examination [34]. Scoring for gastritis and gastritis activity, from mild to severe, was based on the level of infiltrating lymphocytes and neutrophils, respectively. Grading in bacterial density was performed by observing the bacterial count present on gastric epithelia. The presence of large bacterial clumps was considered as severe, whereas a single or small group of two to three organisms was graded from mild to moderate. Other important parameters including atrophy, intestinal metaplasia, and chronic or active gastritis, were also considered important.

Detection of *H. pylori*

PCR detection of *H. pylori* was performed using specific primers targeting 16S rRNA and *ureA* genes (Table 1). For PCR amplification, 800 ng of DNA

Table 1. List of primers used for molecular characterization of *H. pylori*

		Primer	Sequence	Product size	
<i>H. pylori</i> identification	16S rRNA	HP1	CTGGAGAGACTAAGCCCTCC	109 bp	
	UreA	HP2 HP64-F HP64-b	ATTACTGACGCTGATTGTGC TCACCCCAAAAGAGTTAGAC GAAGTGTGAACCGATTTGAA	428bp	
<i>cagA</i>		CAGT-F	ACCCTAGTCGGTAATGGG	variable	
		CAGT-R	GCTTTAGCTTCTGAYACYGC		
		cagA-F	TTGACCAACAACCACAAACCGAAG	183bp	
		cagA-R	CTTCCCTTAATTGCGAGATTCC		
	Western type		CAGT-F	ACCCTAGTCGGTAATGGG	variable
			CAGW-R	TGCCCTACAMCACCSAAACCAC	
			CAGW-F	AAAAATTGACCRACCTCAATC	variable
			CAGT-R	GCTTTAGCTTCTGAYACYGC	
	East Asian type		CAGT-F	ACCCTAGTCGGTAATGGG	variable
			CAGJ-R	GCAATTTTGTTAATCCGGTC	
			CAGJ-F	GCATCAGCAGGTAAAGGAGT	variable
			CAGT-R	GCTTTAGCTTCTGAYACYGC	
<i>vacA</i>	s1 and s2	VA1-F	ATGGAAATACAACAAACACAC	s1=259bp	
		VA1-R	CTGCTTGAATGCGCCAAAC	s2=286bp	
	m1 and m2	VAG-F	CAATCTGTCCAATCAAGCGAG	m1=645bp	
		VAG-R	GCGTCAAATAATTCCAAGG	m2=570bp	
	s1a	SS1-F	GTCAGCATCACACCGCAAC	190bp	
	s1b	SS3-F	AGCGCCATACCGCAAGAG	187bp	
	S1c	SIC-F	CTCGCTTTAGTGGGGCTA	213 bp	
		VA1-R	CTGCTTGAATGCGCCAAAC		

samples were added to a PCR mixture containing 0.5 mM forward and reverse primers, 1.5 mM MgCl₂, 1U of Taq polymerase (Invitrogen, Milan, Italy), 2.5 µl PCR buffer (Qiagen, Germany), and 200 µM of dNTPs. PCR amplifications were performed according to previously described protocols [35,36].

cagA and *vacA* genotyping

cagA status was determined for *H. pylori* positive samples by polymerase chain reaction (PCR) using two different primers pairs as described previously [12,37] (Table 1). The *cagA* gene was typed using two different sets of primers (Table 1) targeting the specific first repeat (FR) and Western second repeat regions (WSR) for the Western type, and the specific FR and East Asian second repeat regions (ESR) for the East Asian type as previously described [12,38]. The total number of repeats present in each region was determined according to amplicon size.

vacA genotyping (s1, s2, m1, m2) was performed as previously described [39,40]. Subtypes were differentiated by amplicon size (Table 1). The s1 region was subtyped by PCR reactions using specific primers as previously described by Atherton *et al.* [40].

Statistical analyses

Descriptive analysis was performed on a total of 201 *H. pylori* positive samples using Statistical Package for Social Science version 17 (SPSS IBM, Chicago, USA). Pearson Chi-square test was applied

to analyze the significant distribution of various genotypes in different groups of patients. Association trends were determined by Goodman and Kruskal’s lambda (λ) test by cross-tabulation.

Results

A total of 450 dyspeptic patients, 274 males and 176 females ranging in age from 10 to 80 years (average age of 38 ± 15.8), were included in the study. Forty-five (10%) patients had previous family history of gastric ulcer and/or gastritis. The clinical symptoms ordinarily found included dyspepsia, malaise, vomiting, stomachache, and epigastrium pain.

Clinical diagnosis along with gastric endoscopic observations identified 305 (67%) cases with different gastroduodenal lesions, whereas 145 (32%) samples from patients who had intact gastric mucosa were considered normal. Among 305 cases, 217 (71%) were diagnosed as gastritis, 45 (14.7%) as duodenal ulcer, and 36 (11.8%) as gastric ulcer, while 6 (2%) samples showed gastric cancer, later confirmed by histology.

Of the 450 samples analyzed, 201 (45%) were positive for *H. pylori* by PCR assays targeting the *ureA* and 16SrRNA genes; these included 45% (98/217) of the identified gastritis cases, 91% (41/45) of the DU cases, 86% (31/36) of the GU cases, and 83% (5/6) of the GC cases. Furthermore, 26 (18%) of the normal group patients were found positive for *H. pylori* (Table 2).

Table 2. Distribution of *cagA* and *vacA* genotypes

Description	Total		Normal		Gastritis		Gastric Ulcer		Duodenal Ulcer		Gastric Cancer	
	N	%	N	%	N	%	N	%	N	%	N	%
Total HP positive	201		26		98		31		41		5	
<i>cagA</i> positive	104	51	3	11	48	49***	23	74***	26	63**	4	80*
• Western type <i>cagA</i>	97		3		46		23		25		4	
• East Asian type <i>cagA</i>	3		0		2		0		1		0	
<i>vacAs1am1</i>	94	47	9	35	42	43*	22	71*	19	46	3	60*
<i>vacA s1am2</i>	40	20	6	23	24	24	1	3	9	22	1	20
<i>vacAs1bm1</i>	11	5	0	0	4	4	3	10	4	10	0	0
<i>vacAs1bm2</i>	13	6	2	8	6	6	2	6	2	5	1	20
<i>vacAs1cm2</i>	4	2	0	0	1	1	1	3	2	5	0	0
<i>vacAs2m1</i>	28	14	9	35**	17	17	0	0	2	5	0	0

*** P < 0.0005, ** P < 0.005, * P < 0.05

H. pylori with different histopathological findings

Since histological changes predict the clinical outcome of disease in the future, association of *H. pylori* with histopathology is important. In this study we applied the Sydney scoring system to quantify the level of bacterial density, gastritis, and gastritis activity to bioptic samples. Histopathology of the samples positive for *H. pylori* revealed that out of 166 samples, 57 had mild and chronic non-specific gastritis while 24 samples exhibited severe gastritis patterns. No specific grade of gastritis was found associated with bacterial density. Other important pathological trends including follicular gastritis with mild to moderate glandular activity (n = 12), mild and chronic gastritis with intestinal metaplasia (n = 17), atrophic gastritis (n = 18), non-atrophic gastritis (n = 22), and chronic gastritis with focal activity (n = 16) were also noticed in *H. pylori* positive samples.

cagA and *vacA* genotyping

In this study the prevalence of *cagA* gene was 52% (104/201), of which 97 were Western types and 3 were East Asian types (Table 2). Four *cagA* positive samples were not successfully typed. As shown in Table 2, the presence of *cagA* gene was observed in 49% (48/98) of the patients with gastritis, 63% (26/41) with DU, 74% (23/31) with GU, and 80% (4/5) with GC ($P < 0.005$), whereas 11% (3/26) of the normal cases were also positive. East Asian type strains were observed in three samples, two of gastritis and one of DU cases.

The *vacA* genotyping revealed the presence of six different allelic variants of the *vacA* gene in our samples. As shown in Table 2, *s1am1* genotype was predominantly detected in 47% (94/201) of *H. pylori* positive cases, followed by *s1am2*, *s2m1*, *s1bm2*, *s1bm1*, and *s1cm2*. Mixed *vacA* genotypes were observed in 11 cases, indicating the presence of more

than one *H. pylori* strain for each case. Detailed analysis revealed that 22 (71%) of the GU and 3 (60%) of the GC cases had *s1am1* genotype ($P < 0.0005$), which was significantly higher than what was seen in the DU and normal cases. Respective percentages of *s1am2* were 24% and 22%, *s1bm1* 4% and 10%, *s1bm2* 6% and 5%, and *s2m1* 17% and 5% in patients with gastritis and DU. Three (60%) GC patients carried *s1am1*, 1(20%) *s1am2* and 1(20%) *s1bm2* (Table 2).

Association and clinical correlation of virulence genes combinations

Table 3 shows that 54 (57%) *H. pylori* strains had the *vacA s1am1/cagA*⁺ combination, while 40 (42%) were *vacAs1am1/cagA*⁻, indicating that the *s1am1* is present in our population irrespective of *cagA* gene expression ($P \geq 0.05$). However, *vacAs1am2* was more significantly associated with *cagA*⁺ (26; 65%) than *cagA*⁻ cases (14; 35%) ($P \leq 0.005$). In contrast, the *s2m1* genotype was more commonly found in *cagA*⁻ strains (21; 75%) than in *cagA*⁺ (7; 25%) ($P \leq 0.0005$). No *s1cm2* genotypes were observed in *cagA*⁺ cases.

Distribution analysis further showed that 17% of the gastritis patients had *s1am2/cagA*⁺ and 16% had *s1am1/cagA*⁺, followed by the other combinations. Interestingly, 68% of the GU and 32% of the DU patients also exhibited the *s1am1/cagA*⁺ combination, followed by the other combinations, as listed in Table 3. Out of five GC cases, two (40%) carried the *s1am1/cagA*⁺ combination while *s1am1/cagA*⁻, *s1am2/cagA*⁺ and *s1bm2/cagA*⁺ were also observed. Interestingly, a number of patients with normal gastric mucosa carried *s2m1/cagA*⁻; however, association of *s2m1* with the *cagA* gene was seen in 3% and 5% of the cases with gastritis and DU respectively ($P < 0.05$) (Table3).

Table 3. Disease association of *cagA* and *vacA* gene combinations

Description	Total		Normal		Gastritis		Gastric Ulcer		Duodenal Ulcer		Gastric Cancer	
	N	%	N	%	N	%	N	%	N	%	N	%
Total HP positive	201		26		98		31		41		5	
Western type <i>cagA/vacAs1am1</i>	54	57	2	3	16	16	21	68	13	32	2	40
Western type <i>cagA/vacAs1am2</i>	26	13**	1	4	17	17	0	0	7	17	1	20
Western type <i>cagA/vacAs1bm1</i>	8	72	0	0	3	3	2	6	3	7	0	0
Western type <i>cagA/vacAs1bm2</i>	1	8	0	0	0	0	0	0	0	0	1	20
Western type <i>cagA/vacAs2m1</i>			0	0	3	3	0	0	2	5	0	0

Discussion

Etiological association of *H. pylori* with gastric ulcer, duodenal ulcer, and gastric adenocarcinoma is well-known. Although the organism is believed to colonize half of the world's population [41], its association with clinical outcomes differs widely. Moreover, its genetic diversity is largely structured in various ethnic groups and geographical locations. In Asia, the prevalence of *H. pylori* infection differs widely in different countries; for example, Pacific Asians such as Chinese, Korean and Japanese populations are considered to be highly susceptible groups due to the highest prevalence of *H. pylori* infection and GC cases in those countries compared to rest of the world. Comparatively, other countries of the region such as Singapore, Malaysia, Taiwan and Vietnam are considered to be at intermediate risk due to lower prevalence *H. pylori* and GC. On the other hand, epidemiology of *H. pylori* in South Asia presents a very distinct view with a low number of GC cases and high rate of *H. pylori* infection, presumably due to the genetic diversity of the pathogen [42]. In spite of the geographic proximity of these countries, differences in prevalence among different ethnic groups and geographic regions have been reported.

In this study, we observed the presence of *H. pylori* infection in 45% of dyspeptic cases of patients who resided in the southern province of Sindh, Pakistan, mainly in Karachi. In 2004, other authors reported a prevalence of *H. pylori* infection in 56% of dyspeptic patients from Karachi [43]; however, in early 2000, Taj *et al.* reported seroprevalence of *H. pylori* in 80% of the cases from the city [27]. Our report and another from Yakoub *et al.* confirm the decreasing trend of *H. pylori* infection overtime in Karachi, which is comparable to trends seen in other South Asian and European countries [44-46]. Interestingly, earlier studies from northern and central parts of Pakistan reported 66% and 84% prevalence rates among dyspeptic patients [25,29], indicating the difference in *H. pylori* infection in geographic niches and ethnic groups in Pakistan. Interestingly *cagA* gene analysis further confirms the above-mentioned observation. We found that 52% of *H. pylori* strains carried the *cagA* gene with the positivity rate of 80% in GC, 74% in GU, 63% DU, and 11% in normal cases, which is comparable with the observations of a previous report from Karachi, Pakistan [47], but conflicts with a different previous study conducted in the northern and central parts of Pakistan which reported a 26% *cagA* positivity rate. A literature search focusing on studies performed in close

geographic proximities revealed that overall *cagA* positivity ratios are still lower in Pakistan compared to those in neighbouring countries. Previous studies reported results similar to ours or a little higher, with percentages in Iran and Afghanistan at 60% and 67%, respectively [48]; however, our typical South Asian neighbours, such as India and Bangladesh, reported much higher rates [49]. Previously we have also identified that antibiotic resistance in *H. pylori*, which correlates with the absence of the *cagA* gene, is more prevalent in Pakistan compared to the prevalence seen in neighboring countries [50], indicating the difference in epidemiological characteristics of this region. The difference in *H. pylori* infection rates and in *cagA* positivity among various Pakistani cities can be related to either the circulation of different strains or to the susceptibility to *H. pylori* infection of the different ethnicities living in Pakistan. Nonetheless, the analysis of only the 3' end of the *cagA* gene might be considered as a major limitation to the study, and it is important to perform PCR for the *cagA* empty site and dot hybridization. It would be worthwhile to study the genetic makeup and ancestry of *H. pylori* strains circulating in these regions.

In this study we observed the pathogenic attributes of *H. pylori*. As expected, *H. pylori* infection was more highly associated with patients with damaged gastric mucosa, such as gastric ulcer (86%) and duodenal ulcer (91%), than with dyspeptic patients with intact gastric mucosa (18%). This observation contrasts earlier studies which report the approximately equal rate of *H. pylori* infection in patients with normal and damaged gastric mucosa [28]. Histological examination of *H. pylori* infected cases further showed the presence of gastric ulcer promoting conditions such as follicular, mild and chronic gastritis with intestinal metaplasia and atrophic gastritis. Apart from the stronger association with diseased cases, *H. pylori* infection was observed only in 45% of the gastritis patients. Given that *H. pylori* was not involved in the induction of gastritis in rest of the patients, it is difficult to predict the other causes. Previous reports suggest that non-*H. pylori* gastritis is primarily present in pediatric patients or others with underlying co-morbidities such as inflammatory bowel disease, autoimmune, and genetic diseases [51]. However, our studied population mainly constituted of adult patients with no known co-morbidities; therefore, it may be plausible that other factors such as fungal and viral infections, excessive intake of NSAIDs or other drugs, and immunological disorders are responsible of inducing gastritis in these

patients, as previously observed in other countries [52].

Understanding the epidemiology of *H. pylori* infection has been greatly facilitated by molecular typing methods which reveal genetic variations in the *vacA* gene and allow the estimation of future clinical outcomes. Low infection rates coupled with stronger clinical associations, high *cagA* positivity, and the predictive change in the gastritis pattern prompted us to scrutinize these samples for various *vacA* genotypes which are directly linked with the virulence stature of *H. pylori*. Previous studies have suggested that *vacA* genotypes s1a and s1b are prominently associated with high toxin activity. Our study on *vacA* genotyping, as expected, revealed significant association of these subtypes, *i.e.*, s1am1, s1bm1 with diseased cases such as gastritis, gastric ulcer and duodenal ulcer when compared with patients who had intact gastric mucosa; however, the situation was more prominent in gastritis cases. Published reports from different geographical regions provide this evidence despite other differences in *H. pylori* genotypes [6], but contrary to their observations, we did not find the s1cm1 type in our studied population.

Another striking feature of our study was the presence of the s2m1 *vacA* genotype in certain gastritis and normal cases. Although s2m1 positive *H. pylori* strains have previously been observed in many countries including Cuba, India, China, South Africa and the Middle East [53-56], they are still regarded as an “alien” in gastroenterology due to their infrequent presence. Such strains are generally considered to be non-toxic [57], which, to some extent justifies their presence in patients with intact mucosa and indicates less likely involvement in disease pathology. However, it is noteworthy that in our study, s2m1 subtypes were also found in gastritis patients, which indicates the diverse epidemiology of gastritis in the Pakistani population. To monitor whether s2m1 positive samples provide some trend, descriptive analysis was performed with respect to various demographical characteristics including age, gender, and the time of sample collection, but no significant association was observed (data not shown). Since these findings are based on PCR methods, it is important to sequence such strains to come to a final conclusion. Our observations, however, warrant in-depth analysis for s2m1 positive *H. pylori* strains in Pakistan and other neighboring countries.

In conclusion, this investigation presents the distribution pattern of the *cagA* and *vacA* genes of *H. pylori* and their association with gastroduodenal

pathologies which could contribute to understanding the trend of *H. pylori* infection in Pakistan.

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